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		DB=PGPB,USPT,USOC,EPAB,JPAB,DWPI,TDBD; PLUR=YES; OP=AND	
☐	L1	(epitope or paratope or tope or minotope or $tope or antigen or immunogen or immunogenic) near5 (presentation or presented or presents or orientation or orients or enhances or improves)	16152
☐	L2	L1 same (loop or cys-cys or cysteine-cysteine or cysteinecysteine or disulfide or di-sulfide)	878
☐	L3	L2 and (pseudomonas or exotoxin or pe-a or exotoxina or exotoxin-a)	603
☐	L4	L2 same (ib or 1b or pseudomonas or exotoxin or pe-a or exotoxina or exotoxin-a)	7
☐	L5	l1.clm.	531
☐	L6	(loop or cys-cys or cysteine-cysteine or cysteinecysteine or disulfide or di-sulfide).clm.	127028
☐	L7	L6 and l5	24
☐	L8	disulfide near3 loop$	1423
☐	L9	L8 near25 (foreign or heterologous or epitope or antigen or immunogen)	34
☐	L10	loop near3 immunogen$	79
☐	L11	280-364	3
☐	L12	$365-399 or (365 near2 299) or 365-399	9369
☐	L13	L12 and loop	1652
☐	L14	L13 and (exotoxin or exotoxina or pe or pseudomonas)	506
☐	L15	L14 and l1	187
☐	L16	$365-399 or (365 near 299) or 365-399	9331
☐	L17	$365-399 or (365 near 399) or 365-399	9347
☐	L18	L17 same loop	103
☐	L19	l18 and $toxin	22
☐	L20	cell.clm. and translocation.clm. and reticulum.clm.	23
☐	L21	cell.clm. and translocation.clm. and reticulum.clm. and loop.clm.	5

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Document ID: US 5541297 A

L8: Entry 64 of 102 File: USPT Jul 30, 1996

DOCUMENT-IDENTIFIER: US 5541297 A
TITLE: Therapeutic conjugates of toxins and drugs

CLAIMS:

1. A hypoimmunogenic immunoconjugate, which comprises an antibody Fab or Fab' fragment that specifically binds to a tumor-associated antigen of a targeted tumor cell or an antigen associated with a protozoan, said tumor associated antigen being on a lymphoma, carcinoma, sarcoma, leukemia or myeloma cell, wherein said antibody fragment is conjugated through a first thiol-binding linker to a drug or modified toxin devoid of a functional receptor-binding domain, and further conjugated through at least a second thiol-binding linker to at least one polysaccharide or polyol group, wherein the antibody thiol groups linked to said linkers are derived from reduction of heavy chain disulfide bonds; and wherein said antigen internalizes said conjugate into the cytoplasm of said targeted cell or microbe.

4. The immunoconjugate of claim 3, wherein said toxin is a modified Pseudomonas exotoxin.

8. A method of producing a hypoimmunogenic immunoconjugate, comprising the steps of:

(a) partially reducing an intact antibody that specifically binds to a tumor-associated antigen of a targeted tumor cell or an antigen associated with a protozoan, said tumor-associated antigen being on a lymphoma, carcinoma, sarcoma, leukemia or myeloma cell, wherein said reduction is effected with a reducing agent for cleaving disulfide groups, in an amount sufficient to generate a plurality of proximal free sulfhydryl groups but insufficient to render immunologically inactive said antibody or to completely cleave the antibody heavy chain, and recovering partially reduced antibody;

(b) enzymatically cleaving said partially reduced antibody with pepsin or papain to generate a F(ab').sub.2 or F(ab).sub.2 fragment, and recovering said fragment; and either

(c) reacting said F(ab').sub.2 or F(ab).sub.2 fragment with at least one polysaccharide or polyol group coupled to a thiol-reactive linker, to conjugate said polysaccharide or polyol group to said fragment, and recovering the resultant conjugate;

(d) cleaving the remaining disulfide groups linking the heavy chains of the product of step (c) with a reducing agent for cleaving disulfide groups, and recovering the resultant polysaccharide- or polyol-conjugated Fab' or Fab fragment having at least one free sulfhydryl group; and

(e) reacting the product of step (d) with a drug or modified toxin devoid of a functional receptor-binding domain and coupled to a thiol-reactive linker, and recovering the resultant hypoimmunogenic immunoconjugate, or

(c') reacting said F(ab').sub.2 or F(ab).sub.2 fragment with a drug or modified toxin devoid of a functional receptor-binding domain and coupled to a thiol-reactive linker, and recovering the resultant conjugate;

(d') cleaving the remaining disulfide groups linking the heavy chains of the

product of step (c') with a reducing agent for cleaving disulfide groups, and recovering the resultant drug- or modified toxin-conjugated Fab' or Fab fragment having at least one free sulfhydryl group; and

(e') reacting the product of step (d') with at least one polysaccharide or polyol group coupled to a thiol-reactive linker, to conjugate said polysaccharide or polyol group to said fragment, and recovering the resultant hypoimmunogenic immunoconjugate.

L8: Entry 66 of 102 File: USPT Feb 20, 1996

DOCUMENT-IDENTIFIER: US 5492893 A
TITLE: Hormone-toxin conjugate compounds

CLAIMS:

1. A compound used for rendering gonadotrophs incapable of secreting gonadotropins, said compound having the general formula ##STR3## wherein T is a toxin group capable of precluding secretion of gonadotropin by said gonadotrophs; X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine; Y is a linking agent; and Z is a substituent selected from the group consisting of Gly-NH.sub.2, ethylamide, and AzA-Gly-NH.sub.2, said compound capable of being conveyed across a cell membrane of said gonadotrophs.

3. A compound of claim 1, wherein Y is selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP),4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),bis-diazobenzidine and glutaraldehyde; and T is a toxin group selected from the group consisting of ricin, modeccin, abrin, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof having at least a toxic domain, wherein said compound is capable of crossing the cell membrane of said gonadotrophs to substantially preclude secretion of hormones by said gonadotrophs.

4. A compound of claim 1, wherein X comprises D-lysine, wherein Y comprises SMPB, wherein Z comprises ethylamide, and wherein T is a toxin group selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins having a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

10. A conjugate of claim 8, wherein said toxin group is selected from the group consisting of ricin, modeccin, abrin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof having at least a toxic domain.

15. A hormone/toxin conjugate of claim 14, wherein said toxic domain is selected from the group consisting of ricin, modecoin, abrin, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin toxic domains; and wherein said modified B-chain is selected from the group consisting of ricin, modeccin, abrin, diphtheria toxin, Pseudomonas exotoxin, and shiga toxin modified B-chains.

17. A conjugate of claim 14, wherein said hormone has the general formula

pyroGlu-His-Trp-Ser-Tyr-X-Leu-Arg-Pro-Z,

wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cystsine, D-cysteine, tyrosine and D-tyrosine; and Z is a substituent selected from the group consisting of Gly-NH.sub.2, ethylamide, and AzA-Gly-NH.sub.2.

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☐	L1	fitzgerald.in. and pseudomonas	90
☐	L2	l1 and (cys or cysteine or loop or disulfide or di-sulfide)	47
		DB=USPT; PLUR=YES; OP=AND	
☐	L3	l1	36
☐	L4	fitzgerald.in. and pseudomonas	36
☐	L5	l4 and (cys or cysteine or loop or disulfide or di-sulfide)	26
☐	L6	l4 and (cys or cysteine or loop or disulfide or di-sulfide).ti,ab,clm.	9
☐	L7	(cys or cysteine or loop or disulfide or di-sulfide).clm. and (pe40 or pea or pe40 or pe-40 or pseudomonas).clm. not l1	102
☐	L8	L7 not l3	102

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☐	L1	fitzgerald.in. and pseudomonas	90
☐	L2	l1 and (cys or cysteine or loop or disulfide or di-sulfide)	47
		DB=USPT; PLUR=YES; OP=AND	
☐	L3	l1	36
☐	L4	fitzgerald.in. and pseudomonas	36
☐	L5	l4 and (cys or cysteine or loop or disulfide or di-sulfide)	26
☐	L6	l4 and (cys or cysteine or loop or disulfide or di-sulfide).ti,ab,clm.	9

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Search Results - Record(s) 1 through 50 of 90 returned.

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2. Document ID: US 6426075 B1

L6: Entry 2 of 9 File: USPT Jul 30, 2002

DOCUMENT-IDENTIFIER: US 6426075 B1
TITLE: Protease-activatable pseudomonas exotoxin A-like proproteins

Abstract Text (1):
This invention provides protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins. The proproteins comprise (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence. The invention also provides methods of using these proproteins for killing target cells.

INVENTOR (1):
Fitzgerald; David J.

Brief Summary Text (2):
Methods and compositions relating to Pseudomonas exotoxin proproteins modified for selective toxicity. The exotoxin is modified to be activated by a desired protease by insertion of a protease activatable sequence in the domain II loop. Activation of the proprotein results in formation of the cytotoxic Pseudomonas exotoxin.

Brief Summary Text (4):
Pseudomonas Exotoxin (PE), which binds and enters mammalian cells by receptor-mediated endocytosis, depends on proteolytic cleavage to generate a C-terminal active fragment which translocates to the cell cytosol, ADP-ribosylates elongation factor 2 and inhibits protein synthesis. Mutant versions of PE which cannot be processed appropriately by cells are non-toxic. Furin has been identified as the intracellular protease responsible for this cleavage. Cleavage occurs between arginine 279 and glycine 280 in an arginine-rich loop located in domain II of the toxin. In biochemical experiments, furin-mediated cleavage is evident only under mildly acidic conditions (pH 5.5). Recently, Garten et al., (EMBO J, 14(11):2424-35 (1995)) have proposed that sequences in the cytoplasmic tail of furin are responsible for its cycling to the cell surface and re-entry through the endosomal compartment. Since PE enters cells via the alpha 2-macroglobulin receptor/Low density lipoprotein receptor-related protein (LRP), it is likely that this receptor delivers PE to an acidic endosomal compartment where it is cleaved by furin. PE is broadly cytotoxic because most mammalian cells and tissues express both LRP and furin. In vivo, the injection of native PE produces profound liver toxicity.

Brief Summary Text (10):

Pseudomonas Exotoxin A ("PE") is translocated into the cytosol after a furin recognition site in domain II is cleaved by furin. Protease-activatable PE-like proproteins are engineered to replace the furin recognition site by a site recognized by a protease made or secreted by a cell targeted for death, for example, a cancer cell. Upon cleavage by the target protease, the PE-like proprotein is translocated into the cytosol where the toxin's ADP-ribosylating activity kills the cell by interfering with polypeptide elongation.

Brief Summary Text (14):
In one aspect this invention provides a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface-receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence.

Brief Summary Text (16):
In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention.

Brief Summary Text (17):
In another aspect, this invention provides a recombinant polynucleotide comprising a nucleotide sequence encoding a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the recombinant polynucleotide is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

Brief Summary Text (18):
In another aspect, this invention provides a method for killing a target cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the cancer cell is, without limitation, a prostate cancer cell, a breast cancer cell or a colon cancer cell.

Brief Summary Text (19):
In another aspect, this invention provides a method for therapeutically treating a subject suffering from cancer comprising administering to the subject a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. More specifically, the PE-like proprotein comprises a protease activatable sequence that is cleavable by an enzyme produced by the cancer cell. The PE-like proprotein can be administered as a pharmaceutical composition.

Drawing Description Text (17):
FIG. 8 is a diagram of Pseudomonas Exotoxin A structure. The amino acid position based on SEQ ID NO:2 is indicated. Domain 1a extends from amino acids 1-252. Domain II extends from amino acids 253-364. It includes a cysteine-cysteine loop formed by cysteines at amino acids 265-287. Furin cleaves within the cysteine-cysteine loop between amino acids 279 and 280. A fragment of PE beginning with amino acid 280 translocates to the cytosol. Constructs in which amino acids 345-364 are eliminated also translocate. Domain Ib spans amino acids 365-399. It contains a cysteine-cysteine loop formed by cysteines at amino acids 372 and 379. The domain can be eliminated entirely. Domain III spans amino acids 400-613. Deletion of amino acid

553 eliminates ADP ribosylation activity. The endoplasmic reticulum sequence, REDKL (SEQ ID NO:33) is located at the carboxy-terminus of the molecule, from amino acid 609-613.

Detailed Description Text (47):
"Pseudomonas exotoxin A" or "PE" is secreted by P. aeruginosa as a 67 kD protein composed of three prominent globular domains (Ia, II, and III) and one small subdomain (Ib) connecting domains II and III. (A. S. Allured et al. (1986) Proc. Natl. Acad. Sci. 83:1320-1324.) Domain Ia of PE mediates cell binding. In nature, domain Ia binds to the low density lipoprotein receptor-related protein ("LRP"), also known as the .alpha.2-macroglobulin receptor (".alpha.2-MR"). (M. Z. Kounnas et al. (1992) J. Biol. Chem. 267:12420-23.) It spans amino acids 1-252. Domain II mediates translocation to the cytosol. It spans amino acids 253-364. Domain Ib has no identified function. It spans amino acids 365-399. Domain III is responsible for cytotoxicity and includes an endoplasmic reticulum retention sequence. It mediates ADP ribosylation of elongation factor 2, which inactivates protein synthesis. It spans amino acids 400-613. PE is "detoxified" if it lacks EF2 ADP ribosylation activity. Deleting amino acid E553 (".DELTA.E553") from domain III detoxifies the molecule. PE having the mutation .DELTA.E553 is referred to herein as "PE .DELTA.E553." Genetically modified forms of PE are described in, e.g., Pastan et al., U.S. Pat. No. 5,602,095; Pastan et al., U.S. Pat. No. 5,512,658, Pastan et al., U.S. Pat. No. 5,458,878 and Pastan et al., U.S. Pat. No. 5,328,984. Allelic forms of PE are included in this definition. See, e.g., M. L. Vasil et al., (1986) Infect. Immunol. 52:538-48. The nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of Pseudomonas exotoxin A are:

Detailed Description Text (48):
II. Protease-Activatable Pseudomonas Exotoxin A-Like Proproteins

Detailed Description Text (50):
Protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins are polypeptides having structural domains organized, except as provided herein, in the same general sequence as the four structural domains of PE, and having certain functions (e.g., cell recognition, cytosolic translocation, cytotoxicity and endoplasmic reticulum retention) also possessed by the functional domains of P.E. More specifically, the general order is: domain Ia, domain II, domain Ib, domain III. However, as described in more detail herein, domain Ia can be eliminated and replaced by a binding protein chemically coupled to the molecule, or, a cell recognition domain can be inserted just before the ER retention sequence in domain III. Domain Ib can be eliminated. Domain II is positioned to the amino-terminal side of domain III.

Detailed Description Text (55):
Protease-activatable Pseudomonas exotoxin-like proproteins comprise an amino acid sequence encoding a "cell recognition domain." The cell recognition domain functions as a ligand for a cell surface receptor. It mediates binding of the protein to a cell. Its purpose is to target the proprotein to a cell which will transport the proprotein to the cytosol for processing. The cell recognition domain can be located in the position of domain Ia of PE. However, this domain can be moved out of the normal organizational sequence. More particularly, the cell recognition domain can be inserted upstream of the ER retention sequence. Alternatively the cell recognition domain can be chemically coupled to the toxin. Also, the proprotein can include a first cell recognition domain at the location of the Ia domain and a second cell recognition domain upstream of the ER retention domain. Such constructs can bind to more than one cell type. See, e.g., R. J. Kreitman et al., Blood 90, pp. 252-259 (1992) Bioconjugate Chem. 3:63-68.

Detailed Description Text (59):
In one embodiment, the cell recognition domain is located in place of domain Ia of PE. In another embodiment, it can be attached to the other moiety of the molecule

through a linker. Engineering studies also show that Pseudomonas exotoxin can be targeted to certain cell types by introducing a cell recognition domain upstream of the ER retention sequence, which is located at the carboxy-terminus of the polypeptide. For example, TGF.alpha. has been inserted into domain III just before amino acid 604, i.e., about ten amino acids from the carboxy-terminus. This chimeric protein binds to cells bearing EGF receptor. Pastan et al., U.S. Pat. No. 5,602,095.

Detailed Description Text (82):
In native Pseudomonas exotoxin A, domain Ib spans amino acids 365 to 399. The native Ib domain is structurally characterized by a disulfide bond between two cysteines at positions 372 and 379. Domain Ib is not essential for cell binding, translocation, ER retention or ADP ribosylation activity. Therefore, it can be entirely re-engineered or eliminated completely. The PE 1b-like domain can be linear or it can include a cysteine-cysteine loop.

Detailed Description Text (203):
The present invention provides Pseudomonas exotoxin A-like proproteins and methods of using them. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

Detailed Description Paragraph Table (4):
SEQUENCE LISTING (1) GENERAL INFORMATION: (iii) NUMBER OF SEQUENCES: 36 (2) INFORMATION FOR SEQ ID NO: 1: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 1839 base pairs (B) TYPE: nucleic acid (C) STRANDEDNESS: single (D) TOPOLOGY: linear (ii) MOLECULE TYPE: DNA (genomic) (ix) FEATURE: (A) NAME/KEY: CDS (B) LOCATION: 1..1839 (D) OTHER INFORMATION: /product= "Pseudomonas exotoxin A" (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1 GCC GAA GAA GCT TTC GAC CTC TGG AAC GAA TGC GCC AAA GCC TGC GTG 48 Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 CTC GAC CTC AAG GAC GGC GTG CGT TCC AGC CGC ATG AGC GTC GAC CCG 96 Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 GCC ATC GCC GAC ACC AAC GGC CAG GGC GTG CTG CAC TAC TCC ATG GTC 144 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met Val 35 40 45 CTG GAG GGC GGC AAC GAC GCG CTC AAG CTG GCC ATC GAC AAC GCC CTC 192 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala Leu 50 55 60 AGC ATC ACC AGC GAC GGC CTG ACC ATC CGC CTC GAA GGC GGC GTC GAG 240 Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu 65 70 75 80 CCG AAC AAG CCG GTG CGC TAC AGC TAC ACG CGC CAG GCG CGC GGC AGT 288 Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 TGG TCG CTG AAC TGG CTG GTA CCG ATC GGC CAC GAG AAG CCC TCG AAC 336 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser Asn 100 105 110 ATC AAG GTG TTC ATC CAC GAA CTG AAC GCC GGC AAC CAG CTC AGC CAC 384 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 ATG TCG CCG ATC TAC ACC ATC GAG ATG GGC GAC GAG TTG CTG GCG AAG 432 Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 CTG GCG CGC GAT GCC ACC TTC TTC GTC AGG GCG CAC GAG AGC AAC GAG 480 Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 ATG CAG CCG ACG CTC GCC ATC AGC CAT GCC GGG GTC AGC GTG GTC ATG 528 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val Met 165 170 175 GCC CAG ACC CAG CCG CGC CGG GAA AAG CGC TGG AGC GAA TGG GCC AGC 576 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 GGC AAG GTG TTG TGC CTG CTC GAC CCG CTG GAC GGG GTC TAC AAC TAC 624 Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 CTC GCC CAG CAA CGC TGC AAC CTC GAC GAT ACC TGG GAA GGC AAG ATC 672 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys Ile 210 215 220 TAC CGG GTG CTC GCC GGC AAC CCG GCG AAG CAT GAC CTG GAC ATC AAA 720 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225 230 235 240 CCC ACG GTC ATC AGT CAT CGC CTG CAC TTT CCC GAG GGC GGC AGC CTG 768 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu 245 250 255 GCC GCG CTG ACC GCG CAC CAG GCT TGC CAC CTG CCG CTG GAG ACT TTC 816 Ala Ala Leu Thr Ala His Gln

Ala Cys His Leu Pro Leu Glu Thr Phe 260 265 270 ACC CGT CAT CGC CAG CCG CGC GGC TGG
GAA CAA CTG GAG CAG TGC GGC 864 Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu
Gln Cys Gly 275 280 285 TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC CTG GCG GCG CGG CTG
TCG 912 Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser 290 295 300
TGG AAC CAG GTC GAC CAG GTG ATC CGC AAC GCC CTG GCC AGC CCC GGC 960 Trp Asn Gln Val
Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly 305 310 315 320 AGC GGC GGC GAC CTG
GGC GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC 1008 Ser Gly Gly Asp Leu Gly Glu Ala
Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 CGT CTG GCC CTG ACC CTG GCC GCC GCC GAG
AGC GAG CGC TTC GTC CGG 1056 Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg
Phe Val Arg 340 345 350 CAG GGC ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG
GTG 1104 Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360
365 AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG GCG GAC 1152 Ser Leu
Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 AGC GGC GAC GCC
CTG CTG GAG CGC AAC TAT CCC ACT GGC GCG GAG TTC 1200 Ser Gly Asp Ala Leu Leu Glu
Arg Asn Tyr Pro Thr Gly Ala Glu Phe 385 390 395 400 CTC GGC GAC GGC GGC GAC GTC AGC
TTC AGC ACC CGC GGC ACG CAG AAC 1248 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr
Arg Gly Thr Gln Asn 405 410 415 TGG ACG GTG GAG CGG CTG CTC CAG GCG CAC CGC CAA CTG
GAG GAG CGC 1296 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg
420 425 430 GGC TAT GTG TTC GTC GGC TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA 1344
Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln 435 440 445 AGC ATC
GTC TTC GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG 1392 Ser Ile Val Phe Gly
Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450 455 460 ATC TGG CGC GGT TTC TAT ATC
GCC GGC GAT CCG GCG CTG GCC TAC GGC 1440 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp
Pro Ala Leu Ala Tyr Gly 465 470 475 480 TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC
CGG ATC CGC AAC GGT 1488 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg
Asn Gly 485 490 495 GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG CCG GGC TTC TAC
1536 Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510
CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG GCG GCG GGC GAG GTC GAA 1584 Arg Thr Ser
Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 CGG CTG ATC GGC CAT
CCG CTG CCG CTG CGC CTG GAC GCC ATC ACC GGC 1632 Arg Leu Ile Gly His Pro Leu Pro
Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 CCC GAG GAG GAA GGC GGG CGC CTG GAG ACC
ATT CTC GGC TGG CCG CTG 1680 Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly
Trp Pro Leu 545 550 555 560 GCC GAG CGC ACC GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC
CCG CGC 1728 Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg 565
570 575 AAC GTC GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1776 Asn
Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln 580 585 590 GCG ATC AGC
GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA CCG CCG 1824 Ala Ile Ser Ala Leu Pro
Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro 595 600 605 CGC GAG GAC CTG AAG 1839 Arg
Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO: 2: (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 613 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE
TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2 Ala Glu Glu Ala Phe Asp Leu
Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 Leu Asp Leu Lys Asp Gly Val Arg Ser
Ser Arg Met Ser Val Asp Pro 20 25 30 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu
His Tyr Ser Met Val 35 40 45 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp
Asn Ala Leu 50 55 60 Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val
Glu 65 70 75 80 Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85
90 95 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser Asn 100 105 110
Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 Met Ser
Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 Leu Ala Arg Asp
Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 Met Gln Pro Thr Leu
Ala Ile Ser His Ala Gly Val Ser Val Val Met 165 170 175 Ala Gln Thr Gln Pro Arg Arg
Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 Gly Lys Val Leu Cys Leu Leu Asp Pro
Leu Asp Gly Val Tyr Asn Tyr 195 200 205 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr
Trp Glu Gly Lys Ile 210 215 220 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu
Asp Ile Lys 225 230 235 240 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly
Ser Leu 245 250 255 Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe
260 265 270 Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly 275 280
285 Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser 290 295 300 Trp
Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly 305 310 315 320 Ser Gly
Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 Arg Leu Ala Leu

Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg 340 345 350 Gln Gly Thr Gly Asn Asp
Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360 365 Ser Leu Thr Cys Pro Val Ala Ala
Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr
Pro Thr Gly Ala Glu Phe 385 390 395 400 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr
Arg Gly Thr Gln Asn 405 410 415 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu
Glu Glu Arg 420 425 430 Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala
Gln 435 440 445 Ser Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450
455 460 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly 465 470 475
480 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly 485 490 495 Ala
Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510 Arg Thr Ser
Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 Arg Leu Ile Gly His
Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 Pro Glu Glu Glu Gly Gly Arg
Leu Glu Thr Ile Leu Gly Trp Pro Leu 545 550 555 560 Ala Glu Arg Thr Val Val Ile Pro
Ser Ala Ile Pro Thr Asp Pro Arg 565 570 575 Asn Val Gly Gly Asp Leu Asp Pro Ser Ser
Ile Pro Asp Lys Glu Gln 580 585 590 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro
Gly Lys Pro Pro 595 600 605 Arg Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO:
3: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 8 amino acids (B) TYPE: amino acid (C)
STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE
DESCRIPTION: SEQ ID NO:3 Asp Arg Val Tyr Ile His Pro Phe 1 5 (2) INFORMATION FOR
SEQ ID NO: 4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 12 amino acids (B) TYPE:
amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi)
SEQUENCE DESCRIPTION: SEQ ID NO:4 Val Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro 1
5 10 (2) INFORMATION FOR SEQ ID NO: 5: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 7
amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii)
MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5 Phe Phe Tyr Thr Pro
Lys Ala 1 5 (2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A)
LENGTH: 9 amino acids (B) TYPE: amino acid

Other Reference Publication (2):
Gray et al; Cloning, Nucleotide Sequence and Expresion in Escherichia coli of Exotoxin A Structural Gene of Pseudomonas aeruginosa; Proc.Natl.Acad.Sci; 81;2645-2649, 1984.*

Other Reference Publication (4):
Allured et al. "Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution," Proc. Natl. Acad. Sci. 83:1320-1324 (1986).

Other Reference Publication (7):
Pastan et al. "Pseudomonas exotoxin: Chimeric toxins," J. of Biol. Chem. 264 (26):15157-15160 (1989).

CLAIMS:

1. A protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hour; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

13. A composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an

exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is substantially un-activatable by fibrin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ WD NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

16. A method for killing a cancer cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence cysteine-cysteine loop is substantially un-activatable by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

☐ 3. Document ID: US 6423513 B1

L6: Entry 3 of 9 File: USPT Jul 23, 2002

DOCUMENT-IDENTIFIER: US 6423513 B1
TITLE: Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins

Abstract Text (1):
This invention provides protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins. The proproteins comprise (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain m of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence. The invention also provides methods of using these proproteins for killing target cells.

INVENTOR (1):
Fitzgerald; David J.

Brief Summary Text (2):
Methods and compositions relating to Pseudomonas exotoxin proproteins modified for selective toxicity. The exotoxin is modified to be activated by a desired protease by insertion of a protease activatable sequence in the domain II loop. Activation of the proprotein results in formation of the cytotoxic Pseudomonas exotoxin.

Brief Summary Text (4):
Pseudomonas Exotoxin (PE), which binds and enters mammalian cells by receptor-mediated endocytosis, depends on proteolytic cleavage to generate a C-terminal active fragment which translocates to the cell cytosol, ADP-ribosylates elongation factor 2 and inhibits protein synthesis. Mutant versions of PE which cannot be processed appropriately by cells are non-toxic. Furin has been identified as the intracellular protease responsible for this cleavage. Cleavage occurs between arginine 279 and glycine 280 in an arginine-rich loop located in domain II of the toxin. In biochemical experiments, furin-mediated cleavage is evident only under mildly acidic conditions (pH 5.5). Recently, Garten et al., (EMBO J, 14(11):2424-35 (1995)) have proposed that sequences in the cytoplasmic tail of furin are responsible for its cycling to the cell surface and reentry through the endosomal compartnent. Since PE enters cells via the alpha 2-macroglobulin receptor/Low density lipoprotein receptor-related protein (LRP), it is likely that this receptor delivers PE to an acidic endosomal compartment where it is cleaved by furin. PE is broadly cytotoxic because most mammalian cells and tissues express both LRP and furin. In vivo, the injection of native PE produces profound liver toxicity.

Brief Summary Text (10):
Pseudomonas Exotoxin A ("PE") is translocated into the cytosol after a furin recognition site in domain II is cleaved by furin. Protease-activatable PE-like proproteins are engineered to replace the furin recognition site by a site recognized by a protease made or secreted by a cell targeted for death, for example, a cancer cell. Upon cleavage by the target protease, the PE-like proprotein is translocated into the cytosol where the toxin's ADP-ribosylating activity kills the cell by interfering with polypeptide elongation.

Brief Summary Text (14):
In one aspect this invention provides a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor; (2) a modified PE translocation domain comprising an amino acid sequence sufficiently homologous to domain II of PE to effect translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the cysteine-cysteine loop is substantially un-activatable by furin; (3) optionally, a PE Ib-like domain comprising an amino acid sequence up to 1500 amino acids; (4) a cytotoxicity domain comprising an amino acid sequence substantially homologous to domain III of PE, the cytotoxicity domain having ADP-ribosylating activity; and (5) an endoplasmic reticulum ("ER") retention sequence.

Brief Summary Text (16):
In another aspect, this invention provides a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention.

Brief Summary Text (17):
In another aspect, this invention provides a recombinant polynucleotide comprising a nucleotide sequence encoding a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the recombinant

polynucleotide is an expression vector further comprising an expression control sequence operatively linked to the nucleotide sequence.

Brief Summary Text (18):
In another aspect, this invention provides a method for killing a target cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. In one embodiment, the cancer cell is, without limitation, a prostate cancer cell, a breast cancer cell or a colon cancer cell.

Brief Summary Text (19):
In another aspect, this invention provides a method for therapeutically treating a subject suffering from cancer comprising administering to the subject a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein of this invention. More specifically, the PE-like proprotein comprises a protease activatable sequence that is cleavable by an enzyme produced by the cancer cell. The PE-like proprotein can be administered as a pharmaceutical composition.

Drawing Description Text (17):
FIG. 8 is a diagram of Pseudomonas Exotoxin A structure. The amino acid position based on SEQ ID NO:2 is indicated. Domain 1a extends from amino acids 1-252. Domain II extends from amino acids 253-364. It includes a cysteine-cysteine loop formed by cysteines at amino acids 265-287. Furin cleaves within the cysteine-cysteine loop between amino acids 279 and 280. A fragment of PE beginning with amino acid 280 translocates to the cytosol. Constructs in which amino acids 345-364 are eliminated also translocate. Domain Ib spans amino acids 365-399. It contains a cysteine-cysteine loop formed by cysteines at amino acids 372 and 379. The domain can be eliminated entirely. Domain III spans amino acids 400-613. Deletion of amino acid 553 eliminates ADP ribosylation activity. The endoplasmic reticulum sequence, REDKL (SEQ ID NO:33) is located at the carboxy-terminus of the molecule, from amino acid 609-613.

Detailed Description Text (45):
"Pseudomonas exotoxin A" or "PE" is secreted by P. aeruginosa as a 67 kD protein composed of three prominent globular domains (Ia, II, and III) and one small subdomain (Ib) connecting domains II and III. (A. S. Allured et al. (1986) Proc. Natl. Acad. Sci. 83:1320-1324.) Domain Ia of PE mediates cell binding. In nature, domain Ia binds to the low density lipoprotein receptor-related protein ("LRP"), also known as the .alpha.2-macroglobulin receptor (".alpha.2-MR"). (M. Z. Kounnas et al. (1992) J. Biol. Chem. 267:12420-23.). It spans amino acids 1-252. Domain II mediates translocation to the cytosol. It spans amino acids 253-364. Domain Ib has no identified function. It spans amino acids 365-399. Domain III is responsible for cytotoxicity and includes an endoplasmic reticulum retention sequence. It mediates ADP ribosylation of elongation factor 2, which inactivates protein synthesis. It spans amino acids 400-613. PE is "detoxified" if it lacks EF2 ADP ribosylation activity. Deleting amino acid E553 (".DELTA.E553") from domain III detoxifies the molecule. PE having the mutation AE553 is referred to herein as "PE .DELTA.E553." Genetically modified forms of PE are described in, e.g., Pastan et al., U.S. Pat. No. 5,602,095; Pastan et al., U.S. Pat. No. 5,512,658, Pastan et al., U.S. Pat. No. 5,458,878 and Pastan et al., U.S. Pat. No. 5,328,984. Allelic forms of PE are included in this definition. See, e.g., M. L. Vasil et al., (1986) Infect. Immunol. 52:538-48. The nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of Pseudomonas exotoxin A are:

Detailed Description Text (46):
PROTEASE-ACTIVATABLE PSEUDOMONAS EXOTOXIN A-LIKE PROPROTEINS

Detailed Description Text (48):
Protease-activatable Pseudomonas exotoxin A-like ("PE-like") proproteins are polypeptides having structural domains organized, except as provided herein, in the

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TGC GTG 48 Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10
15 CTC GAC CTC AAG GAC GGC GTG CGT TCC AGC CGC ATG AGC GTC GAC CCG 96 Leu Asp Leu
Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 GCC ATC GCC GAC ACC
AAC GGC CAG GGC GTG CTG CAC TAC TCC ATG GTC 144 Ala Ile Ala Asp Thr Asn Gly Gln Gly
Val Leu His Tyr Ser Met Val 35 40 45 CTG GAG GGC GGC AAC GAC GCG CTC AAG CTG GCC
ATC GAC AAC GCC CTC 192 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala
Leu 50 55 60 AGC ATC ACC AGC GAC GGC CTG ACC ATC CGC CTC GAA GGC GGC GTC GAG 240
Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu 65 70 75 80 CCG AAC
AAG CCG GTG CGC TAC AGC TAC ACG CGC CAG GCG CGC GGC AGT 288 Pro Asn Lys Pro Val Arg
Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 TGG TCG CTG AAC TGG CTG GTA CCG
ATC GGC CAC GAG AAG CCC TCG AAC 336 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu
Lys Pro Ser Asn 100 105 110 ATC AAG GTG TTC ATC CAC GAA CTG AAC GCC GGC AAC CAG CTC
AGC CAC 384 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120
125 ATG TCG CCG ATC TAC ACC ATC GAG ATG GGC GAC GAG TTG CTG GCG AAG 432 Met Ser Pro
Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 CTG GCG CGC GAT GCC
ACC TTC TTC GTC AGG GCG CAC GAG AGC AAC GAG 480 Leu Ala Arg Asp Ala Thr Phe Phe Val
Arg Ala His Glu Ser Asn Glu 145 150 155 160 ATG CAG CCG ACG CTC GCC ATC AGC CAT GCC
GGG GTC AGC GTG GTC ATG 528 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val
Val Met 165 170 175 GCC CAG ACC CAG CCG CGC CGG GAA AAG CGC TGG AGC GAA TGG GCC AGC
576 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 GGC
AAG GTG TTG TGC CTG CTC GAC CCG CTG GAC GGG GTC TAC AAC TAC 624 Gly Lys Val Leu Cys
Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 CTC GCC CAG CAA CGC TGC AAC
CTC GAC GAT ACC TGG GAA GGC AAG ATC 672 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr
Trp Glu Gly Lys Ile 210 215 220 TAC CGG GTG CTC GCC GGC AAC CCG GCG AAG CAT GAC CTG
GAC ATC AAA 720 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225
230 235 240 CCC ACG GTC ATC AGT CAT CGC CTG CAC TTT CCC GAG GGC GGC AGC CTG 768 Pro
Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu 245 250 255 GCC GCG CTG
ACC GCG CAC CAG GCT TGC CAC CTG CCG CTG GAG ACT TTC 816 Ala Ala Leu Thr Ala His Gln
Ala Cys His Leu Pro Leu Glu Thr Phe 260 265 270 ACC CGT CAT CGC CAG CCG CGC GGC TGG
GAA CAA CTG GAG CAG TGC GGC 864 Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu
Gln Cys Gly 275 280 285 TAT CCG GTG CAG CGG CTG GTC GCC CTC TAC CTG GCG GCG CGG CTG
TCG 912 Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser 290 295 300
TGG AAC CAG GTC GAC CAG GTG ATC CGC AAC GCC CTG GCC AGC CCC GGC 960 Trp Asn Gln Val
Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly 305 310 315 320 AGC GGC GGC GAC CTG
GGC GAA GCG ATC CGC GAG CAG CCG GAG CAG GCC 1008 Ser Gly Gly Asp Leu Gly Glu Ala
Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 CGT CTG GCC CTG ACC CTG GCC GCC GCC GAG
AGC GAG CGC TTC GTC CGG 1056 Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg
Phe Val Arg 340 345 350 CAG GGC ACC GGC AAC GAC GAG GCC GGC GCG GCC AAC GCC GAC GTG
GTG 1104 Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360
365 AGC CTG ACC TGC CCG GTC GCC GCC GGT GAA TGC GCG GGC CCG GCG GAC 1152 Ser Leu
Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 AGC GGC GAC GCC
CTG CTG GAG CGC AAC TAT CCC ACT GGC GCG GAG TTC 1200 Ser Gly Asp Ala Leu Leu Glu
Arg Asn Tyr Pro Thr Gly Ala Glu Phe 385 390 395 400 CTC GGC GAC GGC GGC GAC GTC AGC
TTC AGC ACC CGC GGC ACG CAG AAC 1248 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr
Arg Gly Thr Gln Asn 405 410 415 TGG ACG GTG GAG CGG CTG CTC CAG GCG CAC CGC CAA CTG
GAG GAG CGC 1296 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg
420 425 430 GGC TAT GTG TTC GTC GGC TAC CAC GGC ACC TTC CTC GAA GCG GCG CAA 1344
Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln 435 440 445 AGC ATC
GTC TTC GGC GGG GTG CGC GCG CGC AGC CAG GAC CTC GAC GCG 1392 Ser Ile Val Phe Gly
Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450 455 460 ATC TGG CGC GGT TTC TAT ATC
GCC GGC GAT CCG GCG CTG GCC TAC GGC 1440 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp
Pro Ala Leu Ala Tyr Gly 465 470 475 480 TAC GCC CAG GAC CAG GAA CCC GAC GCA CGC GGC
CGG ATC CGC AAC GGT 1488 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg
Asn Gly 485 490 495 GCC CTG CTG CGG GTC TAT GTG CCG CGC TCG AGC CTG CCG GGC TTC TAC
1536 Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510
CGC ACC AGC CTG ACC CTG GCC GCG CCG GAG GCG GCG GGC GAG GTC GAA 1584 Arg Thr Ser
Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 CGG CTG ATC GGC CAT
CCG CTG CCG CTG CGC CTG GAC GCC ATC ACC GGC 1632 Arg Leu Ile Gly His Pro Leu Pro
Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 CCC GAG GAG GAA GGC GGG CGC CTG GAG ACC
ATT CTC GGC TGG CCG CTG 1680 Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly
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Trp Pro Leu 545 550 555 560 GCC GAG CGC ACC GTG GTG ATT CCC TCG GCG ATC CCC ACC GAC CCG CGC 1728 Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg 565 570 575 AAC GTC GGC GGC GAC CTC GAC CCG TCC AGC ATC CCC GAC AAG GAA CAG 1776 Asn Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln 580 585 590 GCG ATC AGC GCC CTG CCG GAC TAC GCC AGC CAG CCC GGC AAA CCG CCG 1824 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro 595 600 605 CGC GAG GAC CTG AAG 1839 Arg Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO: 2: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 613 amino acids (B) TYPE: amino acid (D) TOPOLOGY: linear (ii) MOLECULE TYPE: protein (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2 Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met Val 35 40 45 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala Leu 50 55 60 Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu 65 70 75 80 Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser Asn 100 105 110 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val Met 165 170 175 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys Ile 210 215 220 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225 230 235 240 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu 245 250 255 Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe 260 265 270 Thr Arg His Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly 275 280 285 Tyr Pro Val Gln Arg Leu Val Ala Leu Tyr Leu Ala Ala Arg Leu Ser 290 295 300 Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro Gly 305 310 315 320 Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335 Arg Leu Ala Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg 340 345 350 Gln Gly Thr Gly Asn Asp Glu Ala Gly Ala Ala Asn Ala Asp Val Val 355 360 365 Ser Leu Thr Cys Pro Val Ala Ala Gly Glu Cys Ala Gly Pro Ala Asp 370 375 380 Ser Gly Asp Ala Leu Leu Glu Arg Asn Tyr Pro Thr Gly Ala Glu Phe 385 390 395 400 Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln Asn 405 410 415 Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu Arg 420 425 430 Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala Gln 435 440 445 Ser Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp Ala 450 455 460 Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr Gly 465 470 475 480 Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly 485 490 495 Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe Tyr 500 505 510 Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val Glu 515 520 525 Arg Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr Gly 530 535 540 Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro Leu 545 550 555 560 Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro Arg 565 570 575 Asn Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu Gln 580 585 590 Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro Pro 595 600 605 Arg Glu Asp Leu Lys 610 (2) INFORMATION FOR SEQ ID NO: 3: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 8 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3 Asp Arg Val Tyr Ile His Pro Phe 1 5 (2) INFORMATION FOR SEQ ID NO: 4: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 12 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4 Val Val Cys Gly Glu Arg Gly Phe Phe Tyr Thr Pro 1 5 10 (2) INFORMATION FOR SEQ ID NO: 5: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 7 amino acids (B) TYPE: amino acid (C) STRANDEDNESS: (D) TOPOLOGY: linear (ii) MOLECULE TYPE: peptide (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5 Phe Phe Tyr Thr Pro Lys Ala 1 5 (2) INFORMATION FOR SEQ ID NO: 6: (i) SEQUENCE CHARACTERISTICS: (A) LENGTH: 9 amino acids

Other Reference Publication (4):
Allured et al., "Structure of exotoxin A of Pseudomonas aeruginosa at 3.0-Angstrom resolution," Proc. Nat'l Acad. Sci., 83:1320-1324 (1986).

Other Reference Publication (7):
Pastan et al., "Pseudomonas exotoxin: Chimeric toxins," J. of Biol. Chem., 264 (26):15157-15160 (1989).

CLAIMS:

1. A recombinant polynucleotide comprising a nucleotide sequence encoding a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain of between 10 and 1500 amino acids that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

4. Document ID: US 6387682 B1

L6: Entry 4 of 9 File: USPT May 14, 2002

DOCUMENT-IDENTIFIER: US 6387682 B1
TITLE: Human osteoclast-derived cathepsin

INVENTOR (7):
Fitzgerald; Lisa M.

Detailed Description Text (34):
Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

CLAIMS:

1. An isolated polypeptide comprising a polypeptide fragment of SEQ ID NO:2 or a polypeptide fragment encoded by the cDNA contained in ATCC Deposit No. 75671, wherein said fragment has cysteine protease activity.

☐ 5. Document ID: US 6383793 B1

L6: Entry 5 of 9 File: USPT May 7, 2002

DOCUMENT-IDENTIFIER: US 6383793 B1
TITLE: Human osteoclast-derived cathepsin

INVENTOR (7):
Fitzgerald; Lisa M.

Detailed Description Text (34):
Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include E. coli, Bacillus subtilis, Salmonella typhimurium and various species within the genera Pseudomonas, Streptomyces, and Staphylococcus, although others may also be employed as a matter of choice.

CLAIMS:

10. An isolated polynucleotide comprising a nucleic acid sequence selected from the group consisting of:

a) a nucleic acid sequence that hybridizes to a polynucleotide consisting of SEQ ID NO:1 or the cDNA contained in ATCC Deposit No. 75671 under hybridization conditions comprising hybridization in a wash buffer consisting of 0.2.times.SSC at 65.degree. C., wherein the nucleic acid sequence encodes a polypeptide with protease activity;

b) a nucleic acid sequence that hybridizes to a polynucleotide consisting of SEQ ID NO:1 under hybridization conditions comprising hybridization in a wash buffer consisting of 0.2.times.SSC at 65.degree. C., wherein the nucleic acid sequence encodes a polypeptide with protease activity;

c) a nucleic acid sequence that hybridizes to a polynucleotide consisting of nucleotides 365 to 1006 of SEQ ID NO:1 under hybridization conditions comprising hybridization in a wash buffer consisting of 0.2.times.SSC at 65.degree. C., wherein the nucleic acid sequence encodes a polypeptide with protease activity; and

d) a nucleic acid sequence that encodes a polypeptide fragment of SEQ ID NO:2 or a polypeptide fragment encoded by the cDNA contained in ATCC Deposit No. 75671, wherein said fragment has cysteine protease activity.

6. Document ID: US 5821238 A

L6: Entry 6 of 9 File: USPT Oct 13, 1998

DOCUMENT-IDENTIFIER: US 5821238 A
TITLE: Recombinant pseudomonas exotoxin with increased activity

Abstract Text (1):
This invention relates to the production and use of recombinant Pseudomonas-derived toxins modified to increase their toxicity and potency in therapy. More particularly, the invention relates to certain deletions in domain II of the amino acid sequence of Pseudomonas exotoxin the domain which relates to the toxin's natural proteolytic processing.

INVENTOR (2):
Fitzgerald; David J.

Brief Summary Text (1):
This invention relates to the production and use of recombinant Pseudomonas-derived toxins modified to increase their toxicity and potency in therapy. More particularly, the invention relates to exotoxins comprising deletions in the amino acid sequence that represent the removal of domain Ia and certain sequences of domain II of Pseudomonas exotoxin.

Brief Summary Text (3):
Toxins attached to growth factors, antibodies and other cell targeting molecules can be used to kill harmful cells bearing specific receptors or antigens (Pastan et al., Cell 47:641 (1986) and Vitetta et al, Science 238:1098 (1987)). One promising source for an effective therapeutic toxin is Pseudomonas exotoxin A. Pseudomonas exotoxin A (PE) is an extremely active monomeric protein (molecular weight 66 kD), secreted by Pseudomonas aeruginosa, which inhibits protein synthesis in eukaryotic cells through the inactivation of elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation (catalyzing the transfer of the ADP ribosyl moiety of oxidized NAD onto EF-2).

Brief Summary Text (7):
This invention discloses improved recombinant Pseudomonas exotoxin molecules that demonstrate higher activities than prior described molecules. Further, the discoveries described here enable one to create PE molecules that are smaller in size, likely to be less immunogenic, that are able to enter the cytosol of target cells, and better able to penetrate the interior of tumors.

Brief Summary Text (9):
The invention includes recombinant Pseudomonas exotoxin molecules in which domain Ia is deleted and no more than the first 27 amino acids from the amino terminal end of domain II have been deleted. A preferred PE molecule begins with a methionine at amino acid position 280 of domain II, comprises the deletion of about amino acids 365 to 380 of domain Ib and includes a substitution of serine at amino acid position 287 in place of cysteine. Preferred molecules also include those that have an amino acid sequence at a carboxyl terminal end of the molecule selected from the group consisting of REDLK (Seq. ID No. 14), REDL (Seq. ID No. 15), and KDEL (Seq. ID No. 16). Exemplary PE molecules may consist essentially of about amino acids 280 to 613 or consist essentially of about amino acids 280 to 364 and 381 to 613.

Drawing Description Text (4):
FIG. 3 is an immunoblot analysis of expressed proteins depicted in FIG. 1 Pseudomonas exotoxin. Molecular masses of the standards are indicated at the left

CLAIMS:

1. A method for impairing tumor growth in a patient comprising administering to the patient intravenously, into a body cavity or into a lumen of an organ a ligand binding agent specific for a tumor cell, fused to a recombinant Pseudomonas exotoxin molecule in which:

(a) domain Ia is deleted;

(b) from 1 to 28 amino acids from the amino terminal end of domain II are deleted;

(c) a methionine occurs at the resultant amino terminal of said molecule; and,

(d) said molecule has increased toxic activity to a target cell as compared to an unmodified PE40.

2. The method of claim 1, wherein the recombinant Pseudomonas exotoxin molecule has amino acids 280 to 364 and 381 to 613 of Sequence ID No: 1 wherein residue 364 is peptide bonded to residue 381.

3. The method of claim 1, wherein the Pseudomonas exotoxin molecule includes a substitution of serine for the amino acid cysteine at position 287 of Sequence ID No: 1.

5. The method of claim 1, wherein amino acids 604-613 of domain III in Sequence ID No: 1 are retained in the Pseudomonas exotoxin molecule.

6. A method for impairing tumor growth in a patient comprising administering to the patient intravenously, into a body cavity or into a lumen of an organ a ligand binding agent specific for a tumor cell, fused to a recombinant Pseudomonas exotoxin molecule (PE) having a deletion in the amino terminal end of domain II such that the molecule is at least 20 times more cytotoxic to target cells than unmodified PE40 in a cytotoxicity assay wherein the cytotoxicity to the target cells of unmodified PE40 and the recombinant PE molecule is measured by assaying against the target cells (i) unmodified PE40 fused to a ligand binding agent specific for the target cells and (ii) the recombinant PE molecule fused to a ligand binding agent specific for the target cells.

☐ 7. Document ID: US 5602095 A

L6: Entry 7 of 9 File: USPT Feb 11, 1997

DOCUMENT-IDENTIFIER: US 5602095 A
TITLE: Recombinant pseudomonas exotoxin with increased activity

Abstract Text (1):
This invention relates to the production and use of recombinant Pseudomonas-derived toxins modified to increase their toxicity and potency in therapy. More particularly, the invention relates to certain deletions in domain II of the amino acid sequence of Pseudomonas exotoxin the domain which relates to the toxin's natural proteolytic processing.

INVENTOR (2):
Fitzgerald; David J.

Brief Summary Text (1):
This invention relates to the production and use of recombinant Pseudomonas-derived toxins modified to increase their toxicity and potency in therapy. More particularly, the invention relates to exotoxins comprising deletions in the amino acid sequence that represent the removal of domain Ia and certain sequences of domain II of Pseudomonas exotoxin.

Brief Summary Text (3):
Toxins attached to growth factors, antibodies and other cell targeting molecules can be used to kill harmful cells bearing specific receptors or antigens (Pastan et al., Cell 47:641 (1986) and Vitetta et al, Science 238:1098 (1987)). One promising source for an effective therapeutic toxin is Pseudomonas exotoxin A. Pseudomonas exotoxin A (PE) is an extremely active monomeric protein (molecular weight 66 kD), secreted by Pseudomonas aeruginosa, which inhibits protein synthesis in eukaryotic cells through the inactivation of elongation factor 2 (EF-2) by catalyzing its ADP-ribosylation (catalyzing the transfer of the ADP ribosyl moiety of oxidized NAD onto EF-2).

Brief Summary Text (7):
This invention discloses improved recombinant Pseudomonas exotoxin molecules that demonstrate higher activities than prior described molecules. Further, the discoveries described here enable one to create PE molecules that are smaller in size, likely to be less immunogenic, that are able to enter the cytosol of target cells, and better able to penetrate the interior of tumors.

Brief Summary Text (9):
The invention includes recombinant Pseudomonas exotoxin molecules in which domain Ia is deleted and no more than the first 27 amino acids from the amino terminal end of domain II have been deleted. A preferred PE molecule begins with a methionine at amino acid position 280 of domain II, comprises the deletion of about amino acids 365 to 380 of domain Ib and includes a substitution of serine at amino acid position 287 in place of cysteine. Preferred molecules also include those that have an amino acid sequence at a carboxyl terminal end of the molecule selected from the group consisting of REDLK (Seq. ID No. 14), REDL (Seq. ID No. 15), and KDEL (Seq. ID No. 16). Exemplary PE molecules may consist essentially of about amino acids 280 to 613 or consist essentially of about amino acids 280 to 364 and 381 to 613.

Drawing Description Text (4):
FIG. 3 is an immunoblot analysis of expressed proteins depicted in FIG. 1 Pseudomonas exotoxin. Molecular masses of the standards are indicated at the left margin.

Detailed Description Text (2):
This invention relates to recombinant Pseudomonas exotoxin molecules having increased cytotoxic activity in which a portion of the amino terminal end of domain II has been deleted. This molecule may be linked or fused to other target molecules so that the improved cytotoxin is targeted to desired cells.

Detailed Description Text (38):
D. Expression and purification of recombinant fusion proteins--Expression of Pseudomonas exotoxin containing fusion proteins was done using the host E. coli strain BL21 (.lambda.DE3) as described previously (Siegall, et al., Proc. Natl. Acad. Sci. USA 85:9738-9742 (1988); Chaudhary, et al., Proc. Natl. Acad. Sci. USA 84:4538-4542 (1987); and Chaudhary, et al, Proc. Natl. Acad. Sci. USA 85:2939-2943 (1988), all incorporated by reference. Cells were incubated for 90 minutes following induction with IPTG (isopropylthiogalactoside). The periplasm fraction was prepared for the mutant proteins from plasmid DF1. For proteins containing the

TGF.alpha. domain, fusion proteins were purified from inclusion bodies as described in Kreitman, et al., supra.

Detailed Description Text (57):
D. Expression and purification of recombinant fusion proteins--Expression of Pseudomonas exotoxin mutant proteins was done using the host BL21 (.lambda.DE3) as described under the previous example. Cells were incubated for 90 minutes following induction with IPTG. The periplasm fraction was prepared for PE35 and NlysPE38 purification. NlysPE38 is a mutant PE protein that contains aa 253-364, 381-613 of PE preceded by an 11 amino acid peptide containing a lysine residue that is easily derivatized. NlysPE38 was purified by sequential use of Q sepharose, Mono Q (HR 5/5; Pharmacia) and TSK-250 (Tosohaas, Montgomeryville, Pa.) columns using Pharmacia LKB Biotechnology Inc. FPLC as described in the prior example. Periplasm containing PE35 was purified by elution from Q sepharose at 0.26-0.30M NaCl in 20 mM Tris pH 7.4. The eluant was injected onto a chelating sepharose column (Pharmacia) that had been 50% saturated with 1 mg/ml CuSO.sub.4 in 50 mM Tris-acetate pH 7.0 containing 1M NaCl. The flow through contained almost pure PE35 that was purified as monomer on a TSK-250 column in PBS containing 10 mM EDTA and 10 mM DTT.

Other Reference Publication (10):
Siegall, C. B. et al., "Functional Analysis of Domains II, Ib, and III of Pseudomonas Exotoxin", Journal of Biological Chemistry, 264: 14256-14261 (1989).

Other Reference Publication (12):
Heimbrook, D. C. et al., "Transforming growth factor .alpha.-Pseudomonas exotoxin fusion protein prolongs survival of nude mice bearing tumor xenografts", Proc. Natl. Acad. Sci. USA, 87: 4697-4701 (1990).

Other Reference Publication (14):
Seetharam, S. et al., "Increased Cytotoxic Activity of Pseudomonas Exotoxin and Two Chimeric Toxins Ending in KDEL", Journal of Biological Chemistry, 266: 17376-17381 (1991).

Other Reference Publication (15):
Kreitman, R. J. et al., "Properties of Chimeric Toxins with Two Recognition Domains: Interleukin 6 and Transforming Growth Factor .alpha. at Different Locations in Pseudomonas Exotoxin", Bioconjugate Chemistry, pp. 63-68 (1992).

Other Reference Publication (16):
Kreitman, R. J. et al., "Rational Design of A Chimeric Toxin: An Intramolecular Location for the Insertion of Transforming Growth Factor .alpha. within Pseudomonas Exotoxin as a Targeting Ligand", Bioconjugate Chemistry, pp. 58-62 (1992).

CLAIMS:

1. An isolated and purified recombinant Pseudomonas exotoxin (PE) molecule having a deletion in the amino terminal end of domain II such that the molecule is at least twenty times more cytotoxic to target cells than unmodified PE40 in a cytotoxicity assay wherein the cytotoxicity to the target cells of unmodified PE40 and the recombinant PE molecule is measured by assaying against the target cells (i) PE40 fused to a ligand binding agent specific for the target cells and (ii) the recombinant PE molecule fused to a ligand binding agent specific-for the target cells.

3. The recombinant PE of claim 1, wherein the molecule includes a substitution of serine for the amino acid cysteine at position 287 of Sequence ID No: 1.

7. An isolated and purified recombinant Pseudomonas exotoxin (PE), molecule in which:

(a) domain la is deleted;

(b) from 1 to 28 amino acids from the amino terminal end of domain II are deleted;

(c) a methionine at the resultant amino terminal of said molecule; and,

(d) said molecule has increased toxic activity to a target cell as compared to an unmodified PF40.

9. The recombinant PE of claim 7, wherein the molecule includes a substitution of serine for the amino acid cysteine at position 287 of Sequence ID No: 1.

8. Document ID: US 4806494 A

L6: Entry 8 of 9 File: USPT Feb 21, 1989

DOCUMENT-IDENTIFIER: US 4806494 A
TITLE: Monoclonal antibody against ovarian cancer cells (OVB-3)

Abstract Text (1):
Monoclonal antibodies are produced which specifically bind to human ovarian cancer cells. These antibodies are conjugated to Pseudomonas exotoxin in order to produce an immunotoxin suitable for the chemotherapeutic treatment of human ovarian cancer.

INVENTOR (2):
Fitzgerald; David J.

Brief Summary Text (2):
Current approaches to cancer chemotherapy and other immunological therapies focus on the use of cell-specific therapeutic agents. Ideally, immunotoxins should discriminate to a high degree between target and non-target cells. The present invention discloses an immmunotoxin conjugate formed between monoclonal antibody OVB-3 (which specifically binds to human ovarian cancer cells) and Pseudomonas exotoxin.

Brief Summary Text (4):
The present invention builds on the discovery disclosed by the same inventors in U.S. Pat. No. 4,545,985, titled "Pseudomonas Exotoxin Conjugate Immunotoxins." This patent is hereby incorporated by reference because it discloses the method used in the present invention for modifying Pseudomonas exotoxin so that the toxin will selectively kill target human tumor cells.

Brief Summary Text (6):
Pseudomonas exotoxin (PE) has been conjugated to a variety of monoclonal antibodies recognizing certain human tumors and to a monoclonal antibody recognizing the human y antigen blood group substance [Richert et al, J. Biol. Chem., 258:8902-8907 (1983); and Fredman et al, J. Biol. Chem., 258:11206-11210 (1983)]. The toxin conjugates specifically kill the appropriate target cells. PE can now be conjugated to a variety of peptides, proteins, and growth factors that react with specific

receptors on cells. These include sarcoma growth factors, melanocyte stimulating hormone (MSH) somatostatin, glucagon, insulin, transferrin, low density lipoprotein, calcitonin, alpha.sub.2 -macroglobulin, and lysine-bradykinin. Pseudomonas exotoxin is particularly preferable to other toxins (such as ricin or diphtheria toxin) because large amounts are easily prepared, because humans do not usually have neutralizing antibodies against it (as is the case with diphtheria toxin), and because it does not need to be separated into subunits before being conjugated (as does ricin toxin).

Detailed Description Text (2):
Pseudomonas exotxin (PE) is a known and readily available toxin isolated from Pseudomonas aeruginosa. The particular exotoxin used in this invention is commercially available through the Swiss Serum Company.

Detailed Description Text (5):
Monoclonal antibody OVB-3 is produced by conventional methods. In general, mice are immunized with OVCAR-3, a human ovarian cancer cell line [described in Hamiliton et al, Cancer Research, 43:5379-5389 (1983)]. Spleen cells from the immunized mouse are then fused with a myeloma cell line, thus forming hybridomas which are capable of producing monoclonal antibodies which specifically bind to human ovarian cancer cells. One such Mab, OVB-3, is highly specific for human ovarian cancer, and is used to instruct the Pseudomonas exotoxin conjugate.

Detailed Description Text (7):
Pseudomonas exotoxin-monoclonal antibody OVB-3 conjugates (PE-OVB-3) are constructed either using a disulfide exchange reaction or by forming a thioether bond. Generally, PE is treated with 2-iminothiolane (formally, methyl-4-mercapto-butyrimidate) in order to introduce two thiol groups per molecule of toxin. This step is optimally conducted in 0.15 M KPO.sub.4 (pH 8.0), 1 mM EGTA. Derivatized PE from the above step is then reacted with dithiobis(2-nitrobenzoic acid), DTNB. Purified OVB-3 is also treated with 2-iminothiolane in order to introduce one sulfhydryl group per molecule. The treated antibody is then mixed with excess treated PE at pH 8.0 and allowed to incubate for 2 hours at 23oC. At the end of the reaction, the pH is adjusted to 7.0 and cysteine is added to displace the TNB from any PE molecules that have not formed conjugates or that have one unreacted --SH group.

CLAIMS:

2. An immunotoxin conjugate for the chemotherapeutic alleviation of human ovarian cancer comprising a monoclonal antibody OVB-3 which specifically binds to ovarian cancer cells bonded to Pseudomonas exotoxin.

3. An immunotoxin conjugate comprising Pseudomonas exotoxin (PE) bound to a monoclonal antibody which specifically binds to ovarian cancer cells wherein said PE is modified by treatment with 2-iminothiolane followed by treatment with dithiobis(2-nitrobenzoic acid) and wherein said monoclonal antibody is modified by treatment with a reagent selected from the group consisting of 2-imminothiolane and m-maleimidobenzoyl N-hydroxysuccinimide ester under conditions which effect the formation of a disulfide or thioether bond between said PE and said monoclonal antibody.

4. A composition of matter comprising a monoclonal antibody of claim 1 conjugated with a Pseudomonas exotoxin.

9. Document ID: US 4545985 A

L6: Entry 9 of 9 File: USPT Oct 8, 1985

DOCUMENT-IDENTIFIER: US 4545985 A
TITLE: Pseudomonas exotoxin conjugate immunotoxins

Abstract Text (1):
A method of modifying Pseudomonas exotoxin (PE) with methyl-4-mercaptobutyrimidate is disclosed so that after conjugating the exotoxin to a monoclonal antibody (ab) such as the antibody to the transferrin receptor, the PE-ab conjugate becomes a highly potent immunotoxin suitable for use against human tumor cells. This same method has been used to conjugate PE to epidermal growth factor (EGF) to create a highly potent growth factor-toxin conjugate for use against cells having large numbers of EGF receptors. Also disclosed are the immunotoxin conjugates for Pseudomonas exotoxin coupled to anti-TFR (antibody to the transferrin receptor) and anti-TAC (antibody to the human T-cell growth factor receptor) and to EGF.

INVENTOR (3):
Fitzgerald; David J.

Brief Summary Text (4):
Current approaches to cancer chemotherapy and other immunological therapies focus on the use of cell-specific antibodies bonded to immunotoxins in order to kill specific populations of human cells. Ideally, immunotoxins should discriminate to a high degree between target and non-target cells. The critical point, then, is the development of immunotoxins that are highly toxic for specific populations of cells. The present invention details a new class of immunotoxins employing a monoclonal antibody, recognizing a specific human cell receptor, bonded to Pseudomonas toxin. Pseudomonas exotoxin (PE) is modified with methyl-4-mercaptobutyrimidate (MMB) so that, by itself, the toxin exhibits very little toxicity; coupling the modified toxin to a monoclonal antibody, however, transforms the toxin into a highly potent immunotoxin.

Brief Summary Text (5):
Other toxins have been modified in order to produce a suitable immunotoxin. The two best known are ricin toxin and diptheria toxin. However, both of these toxins must be cleaved and the A-chain subunits purified prior to bonding with suitable antibodies. With Pseudomonas exotoxin, the cleavage step is unnecessary. In addition, cleavage of ricin or diptheria toxins into A and B chains removes the portion of the molecule containing residues important for transport into the cytosol of the cell. In contrast, when Psuedomonas exotoxin is modified, no part of the molecule is removed; coupling the exotoxin to a suitable monoclonal antibody produces a very potent cell-specific and easily internalized toxin.

Detailed Description Text (2):
A method has been developed to chemically modify Pseudomonas exotoxin so that the exotoxin can be coupled with growth factors, antibodies, and other biologically active molecules so that this toxin will selectively kill target human tumor cells or other types of cells displaying specific molecules on their cell surface.

Detailed Description Text (3):
PE has recently been conjugated to a variety of monoclonal antibodies recognizing certain human tumors (Cetus Corporation) and to a monoclonal antibody recognizing the human H Type 1 blood group substance [Richert et al. (1983) J. Biol. Chem.,

258:8902-8907, and Fredman et al. (1983) J. Biol. Chem., 258:11206-11210]. The toxin-conjugates specifically kill appropriate target cells. PE can now be coupled to a variety of peptides, proteins and growth factors that react with specific receptors on cells. These include sarcoma growth factors, malanocyte stimulating hormone (MSH), somatostatin, glucogon, insulin, transferrin, low density lipoprotein, calcitonin, .alpha..sub.2 -macroglobulin, and lysine bradykinin. Conjugates with MSH and lysine bradykinin have already been prepared and show some biological activity. Pseudomonas exotoxin is particularly preferable to other toxins (such as ricin or diptheria toxin) because it is easily prepared in large amounts and because humans do not contain the antibodies to neutralize it (as is the case with diptheria toxin) and because it does not have to be separated into subunits before being conjugated.

Detailed Description Text (5):
Pseudomonas exotoxin (PE) is a known and readily available toxin isolated from Pseudomonas aeruginosa. The particular exotoxin used in this invention was the gift of Dr. S. A. Leppla, USAMRIID, Fort Detrick, Md.

Detailed Description Text (10):
Pseudomonas exotoxin-monoclonal antibody conjugates (PE-Ab) are constructed either using a disulfide exchange reaction or by forming a thioether bond. For a particular regimen used for a specific Mab, see Examples 1-3. Generally, however, PE is treated with methyl-4-mercaptobutyrimidate (MMB) in order to introduce two thiol groups per molecule of toxin. This step is optimally conducted in 10 mM KPO.sub.4 (pH 8.5). Derivatized PE from the above step is then reacted with dithiobis(2-nitrobenzoic acid) (DTNB). Purified antibody is also treated with MMB in order to introduce slightly more than one thiol group per molecule. The treated antibody is then mixed with excess treated PE and allowed to incubate for 2 hrs at 23.degree. C.

Detailed Description Text (51):
Pseudomonas exotoxin modified with MMB need not be coupled to a monoclonal antibody in order to be biologically effective. At this point, no other toxin can be so modified and remain effective without killing the host animal. For example, less than 1 .mu.g of unmodified toxin is lethal I.P. When Pseudomonas exotoxin was modified and coupled to EGF, nude mice were killed by 20-50 .mu.g of the conjugate. Autopsy showed that they died from liver failure. This is because the liver has a large number of EGF receptors. On the other hand, when Pseudomonas exotoxin was coupled to cysteine by the same chemical reaction, animals given 0.3-0.5 mg of toxin exhibited no signs of toxicity. These experiments show the low toxicity of the chemically modified toxin when coupled to an inert molecule and how it can be coupled to a growth factor to kill receptors containing cells of the liver.

Other Reference Publication (2):
Schreiber, Barbara Mildred, Diss. Abst., vol. 41, No. 12, Jun. 1981, p. 1405b, "In Vitro Toxicity of Antibody Pseudomona, avergihusu Exotoxin A Conjugates".

CLAIMS:

1. A method for the chemotherapeutic alleviation of cancer in animals consisting essentially of injecting a chemotherapeutically alleviating amount of an immunotoxin conjugate which comprises a modified Pseudomonas exotoxin (PE) and a modified cancer cell binding protein wherein said PE is modified by treatment with methylmercaptobutyrimidate (MMB) followed by treatment with dithiobis(2-nitrobenzoic acid) and wherein said cancer cell binding protein is modified by treatment with a reagent selected from the group consisting of MMB and m-maleimidobenzoyl N hydroxy-succinimide ester under conditions which effect the formation of a disulfide or thioether bond between said PE and said cancer cell-binding protein.

2. The method of claim 1 wherein said Pseudomonas exotoxin is modified with methyl-4-mercapto-butyrimidate and dithiobis(2-nitrobenzoic acid).

3. The method of claim 1 wherein said cancer cell-binding protein is selected from one member of the group consisting of anti-TAC monoclonal antibody, anti-TFR monoclonal antibody, epidermal growth factor, and cysteine.

6. A process for the production of a cancer cell-specific immunotoxin comprising chemically bonding modified Pseudomonas exotoxin (PE) to a modified monoclonal antibody (Ab) wherein PE is modified by treatment with methylmercaptobutyrimidate and dithiobis(2-nitrobenzoic acid) and Ab is modified by one reagent selected from the group consisting of methylmercaptobutyrimidate and m-maleimidobenzoyl N hydroxysuccinimide ester under conditions which effect the formation of a disulfide or thioether bond between said PE and said Ab.

7. The process of claim 6 in which said Pseudomonas exotoxin is modified by treatment with mercaptobutyrimidate under conditions in which 2 moles of sulhydryl are used for every mole of toxin.

10. An immunotoxin conjugate consisting essentially of Pseudomonas exotoxin (PE) bound to a cancer cell binding protein wherein PE is modified by treatment with methylmercaptobutyrimidate (MMB) followed by treatment with dithiobis(2-nitrobenzoic acid) and wherein said cancer cell binding protein is modified by treatment with a reagent selected from the group consisting of MMB and m-maleimidobenzoyl N-hydroxysuccinimide ester under conditions which effect the formation of a disulfide or thioether bond between said PE and said cancer cell-binding protein.

12. The immunotoxin conjugate of claim 10 wherein said Pseudomonas exotoxin is modified by treatment with methyl-mercaptobutyrimidate and followed by treatment with dithiobis(2-nitrobenzoic acid) under conditions in which 2 moles of sulfhydryl are used for every mole of toxin.

13. The immunotoxin conjugate of claim 10 wherein said cancer cell-binding protein is selected from the group consisting of anti-TAC monoclonal antibody, anti-TFR monoclonal antibody, epidermal growth factor, and cysteine.

14. An immunotoxin conjugate comprising modified Pseudomonas exotoxin (PE) and a modified monoclonal antibody (Ab) wherein PE is modified by treatment with methylmercaptobutyrimidate (MMB) followed by dithiobis(2-nitrobenzoic acid) wherein the monoclonal antibody is modified with a reagent selected from one member of the group consisting of MMB and m-maleimidobenzoyl N hydroxy-succinimide ester under conditions which effect the formation of a disulfide bond or thioether bond between PE and Ab.

15. An immunotoxin conjugate of claim 14 wherein said Pseudomonas exotoxin is modified by treatment with methylmercaptobutyrimidate followed by treatment with dithiobis(2-nitrobenzoic acid) under conditions in which 2 moles of sulfhydryl are used for every mole of toxin.

16. An immunotoxin comprising Pe-B-Ab wherein PE is Pseudomonas exotoxin, Ab is a monoclonal antibody selected from the group consisting of anti-TAC and anti-TFR, and B is a sulfhydryl group bridging agent wherein PE is treated with methyl-4-mercaptobutyrimidate (MMB) followed by treatment with dithiobis(2-nitrobenzoic acid) and Ab is treated with MMB under conditions which effect the formation of a disulfide or thioether bond between said PE and said Ab.

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☐ 1. Document ID: US 6881718 B1

L6: Entry 1 of 9 File: USPT Apr 19, 2005

DOCUMENT-IDENTIFIER: US 6881718 B1
TITLE: Disulfide conjugated cell toxins and methods of making and using them

Abstract Text (1):
The invention pertains to the discovery of novel disulfide linked cell toxins which can ablate NK-1 receptor expressing cells. These toxins are used as pharmaceutical compositions for the ablation of NK1 receptor expressing cells and comprise a substance P (SP)-Pseudomonas exotoxin disulfide linked conjugate. The invention also includes methods of making and using these toxins and pharmaceutical compositions.

INVENTOR (1):
FitzGerald; David J.

Brief Summary Text (9):
The invention provides a method of making a cell toxin comprising reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups. In this method the Pseudomonas exotoxin translocation domain sulfhydryl group can be located within ten amino acid residues of the amino terminus or at the amino terminus. The Pseudomonas exotoxin translocation domain sulfhydryl group can be a cysteine residue or an equivalent, e.g., a peptidomimetic, an analog, a conservative substitution variation. The Pseudomonas exotoxin translocation domain can be covalently linked to the Pseudomonas exotoxin ADP-ribosylation domain, for example, the covalent linkage between the Pseudomonas exotoxin translocation domain and the Pseudomonas exotoxin ADP-riboslyation domain can be a peptide bond, or equivalent structure, as discussed below. The Pseudomonas exotoxin translocation domain can comprise an amino acid sequence as set forth in SEQ ID NO:1. The Pseudomonas exotoxin ADP-riboslyation domain can comprise an amimo acid sequence as set forth in SEQ ID NO:2.

Brief Summary Text (10):
The invention also provides a pharmaceutical composition for the ablation of NK1 receptor expressing cells. The pharmaceutical composition comprises a cell toxin and a pharmaceutically acceptable excipient, wherein the cell toxin is a substance

P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-riboslyation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenozate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups. The Pseudomonas exotoxin translocation domain sulfhydryl group can located within ten amino acid residues of the amino terminus or it can be located at the amino terminus. The Pseudomonas exotoxin translocation domain sulfhydryl group can be a cysteine residue or equivalent residue, as discussed below. The Pseudomonas exotoxin translocation domain can covalently linked to the Pseudomonas exotoxin ADP-riboslyation domain, for example, the covalent linkage between the Pseudomonas exotoxin translocation domain and the Pseudomonas exotoxin ADP-riboslyation domain can be a peptide bond. The Pseudomonas exotoxin translocation domain can comprise an amino acid sequence as set forth in SEQ ID NO:1. The Pseudomonas exotoxin ADP-riboslyation domain can comprise an amino acid sequence as set forth in SEQ ID NO:2. The cell toxin and pharmaceutically acceptable excipient can be suitable for administration intrathecally, subdurally, directly into the brain parenchyma, intraventricularly, or directly into a tumor (or systemic administration) for treatment of cancer cells that express a receptor that binds to SP (e.g., NK-1 receptor).

Brief Summary Text (11):
The invention also provides a method for ablating an NK1 receptor expressing cell in a patient comprising administering a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to ablate an NK1 receptor expressing cell, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-riboslyation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups. The ablated NK1 receptor expressing cell can be dorsal horn cells, neostriatalcells or other brain parenchyma cells. The ablated NK1 receptor expressing cell can also be inflammatory or immune cells, respiratory cells, genitourinary cells or gastrointestinal tract cells or tumore cells that express a receptor that binds SP (e.g., NK-1 receptor).

Brief Summary Text (12):
The invention also provides a method of treating chronic pain without significantly affecting basal nociceptive responses comprising administering a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to treat chronic pain without significantly affecting basal nociceptive responses, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after

the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups. The cell toxin can be administered, for example, in the form of a pharmaceutical composition, to epineurium cells, perineurium cells, nerve ganglia, nerve sheathes, nerve linings, meninges, pia mater cells, arachnoid membrane cells, dura membrane cells, cells lining a joint or brain or spinal cord parenchymal cells or tumor cells expressing the proper receptor. The pharmaceutical composition can be administered intrathecally or injected directly into spinal cord or brain parenchymal cells, or joint spaces, or intravenously. For example, the pharmaceutical composition can be delivered into the subarachnoid space through implanted or external intrathecal or epidural pumps.

Detailed Description Text (2):
This invention is the discovery of a of a novel disulfide linked cell toxin conjugate comprising a modified substance P ("SP") peptide and a modified Pseudomonas exotoxin polypeptide. This novel cell toxin conjugate is particularly effective in ablating NK-1 receptor-expressing cells. Killing such cells in an effective means of treating a variety of conditions, particularly, chronic pain or tumors that express a receptor that binds SP, e.g., NK-1 receptors.

Detailed Description Text (5):
The modified Pseudomonas exotoxin ("PE") polypeptide comprises a Pseudomonas exotoxin cell translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain. The Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group; one exemplary translocation domain has only one cysteine group (or equivalent, e.g., a peptidomimetic); e.g., at the residue corresponding to position 287 of the naturally expressed PE, as described below.

Detailed Description Text (16):
The terms "polypeptide" and "peptide" including "Pseudomonas exotoxin translocation domain, "Pseudomonas exotoxin ADP-ribosylation domain" and "substance P" (or "SP") peptide includes polypeptides and peptides having an activity and a structural characteristic which substantially corresponds to their corresponding polypeptides. These include "analogs," "conservative variants," "peptidomimetics" and "mimetics" with structures and activity which substantially correspond to exemplary sequences. For example, an exemplary, modified Pseudomonas exotoxin translocation domain comprises an amino acid sequence as set forth in SEQ ID NO:1 (with only one cysteine residue, as discussed below) and an exemplary Pseudomonas exotoxin ADP-ribosylation domain comprises an amino acid sequence as set forth in SEQ ID NO:2 (residues 400 to 613 of SEQ ID NO:6). The amino acid sequences set forth in the present application use the conventional practice wherein the amino group is presented to the left (the N-terminus) and the carboxyl group to the right (the C-terminus) of each amino acid residue.

Detailed Description Text (19):
Pseudomonas exotoxin translocation domain variants or mimetics thereof are within the scope of the invention if they are capable of translocating through an endosmal/microsmal membrane into the cytosol (means for such determinations are well known in the art, see e.g., Theuer (1994) Biochemstry 33:5894-5900). A Pseudomonas exotoxin ADP-ribosylation domain variant or mimetic thereof is within the scope of the invention if, after translocation to the cytosol, can inhibit protein synthesis by ADP-ribosylating elongation factor 2 (means for such determinations are well known in the art, see, e.g. Zdanovsky (1993) J. Biol. Chem. 268(29):21791-21799; or, the method of Collier and Kandel, as described in Mansfield (1998)Bioconjugate Chem. 7:557-583, p. 558). The cell killing effectiveness of the cell toxin can be determined using any cytotoxicity assay, many of which are well known in the art (e.g., cell death quantitated using the MTT method of Mosmann (1983) J. Immunol. Meth. 65:55-63).

Detailed Description Text (21):
The terms "mimetic" and "peptidomimetic" refer to a synthetic chemical compound which has substantially the same structural and/or functional characteristics of the Pseudomonas exotoxin translocation domain, Pseudomonas exotoxin ADP-ribosylation domain and "SP" polypeptides and peptides of the invention. The mimetic can be either entirely composed of synthetic, non-natural analogues of amino acids, or, is a chimeric molecule of partly natural peptide amino acids and partly non-natural analogs of amino acids. The mimetic can also incorporate any amount of natural amino acid conservative substitutions as long as such substitutions also do not substantially alter the mimetic's structure and/or activity. As with polypeptides of the invention which are conservative variants, routine experimentation (as discussed herein) will determine whether a mimetic is within the scope of the invention, i.e., that its structure and/or function is not substantially altered. Polypeptide mimetic compositions can contain any combination of nonnatural structural components, which are typically from three structural groups: a) residue linkage groups other than the natural amide bond ("peptide bond") linkages; b) non-natural residues in place of naturally occurring amino acid residues; or c) residues which induce secondary structural mimiery, i.e., to induce or stabilize a secondary structure, e.g., a beat turn, gamma turn, beta sheet, alpha helix conformation, and the like.

Detailed Description Text (22):
The term "llinked" means polypeptide domains or individual residues are joined, "linked" by means other than natural peptide bonds. These include, e.g., electrostatic (e.g., ionic, van der Waals or hydrogen bonds) or chemical means. For example, the cell toxins of the invention comprise a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain. Polypeptide domains or individual peptidomimetic residues can be joined by peptide bonds or other chemical bonds or coupling means, such as, e.g., glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, e.g., ketomethylene (e.g., --C(.dbd.O)--CH.sub.2 -- for --C(.dbd.O)--NH--), aminomethylene (CH.sub.2 --NH), ethylene, olefin (CH.dbd.CH), ether (CH.sub.2 --O), thioether (CH.sub.2 --S), tetrazole (CN.sub.4 --), thiazole, retroamide, thioamide, or ester (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, N.Y.).

Detailed Description Text (49):
Pseudomonas exotoxin A

Detailed Description Text (50):
Following SP-NK-1 receptor binding on the cell surface, the ligand-receptor complex is internalized. This makes the NK-1 receptor a rational target for toxin-derived therapeutics. Pseudomonas exotoxin is a member of a protein family functionally characterized and a "reverse internalized predator." Toxins in this class act in the cytosol of mammalian cells to inhibit protein synthesis and cause cell death at very low concentrations. These toxins use the endocytosis pathway to gain entry to intracellular sorting vesicles, where they unfold and translocated into the cytosol. After translocation they refold and enzymatically inhibit protein synthesis. Because of high enzymatic catalytic rates and apparent resistance of these toxins to proteosome degradation, only one or a few molecules need to reach to cytosol to be effective.

Detailed Description Text (51):
Naturally expressed Pseudomonas exotoxin A can be used as a model to design toxin polypeptide domains to be used in the toxin conjugates of the invention. The naturally expressed toxin is secreted by Pseudomonas aeruginosa as a 67 kD protein

ADP → 400-613

that an initiating methionine is used as residue in place of the gylcine at residue 280 (in naturally occurring SEQ ID NO:6) (see also Theur (1993) Cancer Res. 53:340-347).

Detailed Description Text (57):
An exemplary Pseudomonas exotoxin ADP-ribosylation domain comprises an amino acid sequence as set forth in SEQ IN NO:2, which corresponds to amino acids 400 to 613 of SEQ ID NO:6.

Detailed Description Text (58):
The polypeptides of the invention include conservative variants, mimetics and analogs which have modified amino acid sequences such that the change(s) do not substantially alter the variants activity. Thus, a Pseudomonas exotoxin translocation domain with conservative variants is within the scope of the invention if it is capable of translocating through an endosomal/microsomal membrane into the cytosol (means for such determinations are well known in the art, see, e.g., Theuer (1994) supra).

Detailed Description Text (59):
A Pseudomonas exotoxin ADP-ribosylation domain with conservative variants is within the scope of the invention if, after translocation to the cytosol, can inhibit protein synthesis by ADP-ribosylating elongation factor 2 (means for such determinations are well known in the art, see, e.g. Zdanovsky (1993) supra).

Detailed Description Text (60):
The nucleotide sequence (SEQ ID NO:5) and deduced amino acid sequence (SEQ ID NO:6) of Pseudomonas areoginosa exotoxin A are:

Detailed Description Text (62):
Any functional Pseudomonas polypeptide translocation domain can be used in the toxin conjugate of the invention. The functionality of a translocation domain can be tested as a function of the domain's ability to mediate and gain access to the cytosol (because access first requires binding to the cell, these assays are also useful to determine whether the SP domain is functioning, i.e., binding to an NK-1 expressing cell). In one method, access to the cytosol is determined by detecting the physical presence of the toxin conjugate in the cytosol. For example, the toxin conjugate can be labeled with a composition that is detectable after the toxin conjugate gains entry inside a cell. Then, the cytosolic fraction is isolated (the endosomal compartment can also be separately isolated) and the amount of label in the cytosol fraction determined. Detecting label in the cytosol indicates that the translocation domain is functional and that the toxin conjugate has gained access to the cytosol.

Detailed Description Text (64):
Any functional Pseudomonas polypeptide translocation domain can be used in the toxin conjuate of the invention. The functionality of a domain III having ADP ribosylation activity can also be tested in a variety of ways. For example, cells can be seeded in tissue culture plates and expoed to the toxin conjuate. ADP ribosylation activity is determined as a function of inhibition of protein synthesis by, e.g., monitoring the incorporation of .sup.3 H-leucine.

Detailed Description Text (65):
Recombinant Expression of Pseudomonas Polypeptide Domains

Detailed Description Text (66):
The functional Pseudomonas polypeptide translocation and ADP ribosylation domains can be expressed in any in vitro or in vivo recombinant system. In one exemplary system, Pseudomonas polypeptide translocation and ADP ribosylation domains are designed as a single recombinant molecule based on the naturally expressed toxin (SEQ ID NO:6). In one exemplary design the recombinant molecules uses an initiating

methionine in place of glycine at residue 280 (of SEQ ID NO:6), retains the cysteine of residue position 287, and eliminates the disulfide bond at residues 374 to 379; the resulting polypeptide is SEQ ID NO:1.

Detailed Description Text (74):
The invention provides disulfide linked cell toxin conjugates comprising a modified SP peptide and a modified PE translocation domain linked to a PE ADP-ribosylation domain. chemical conjuation is advantageous over synthesis by, e.g., entirely recombinant techniques, because a better defined homogenous product is obtained (recombinant expression in vivo, e.g., mammalian cells or bacteria, in contrast, commonly results in mixed products due to posttranslational modification, degradation, and the like). In the present invention, conjugates are made by "disulfide exchange." To ensure that the ratio of SP to PE in each conjugate molecule is 1:1, the SP peptide and the Pseudomonas exotoxin component of the toxin conjugate each have only one reactive sulfhydryl group. The sulfhydryl group for disulfide exchange can be in the form of a cysteine (or equivalent, see above) residue.

Detailed Description Text (76):
In one exemplary protocol, SP-TNB is added to the pseudomonas exotoxin component (having only one reactive sulfhydryl group) in a 8:1 molar ratio for an overnight incubation at 4C. Progress of the reaction is monitored, e.g., by measuring absorbance at OD 412 nm. SP-TNB can be purified, e.g., by HPLC. The SP-PE pharmaceuticals of the invention have less than 2% thionitro benzoate groups.

Detailed Description Text (77):
SP-TNB is dissolved in 20% DMSO (e.g., 2 mg SP-TNB in 20 microliters DMSO). Then 1.0 milliliter of 0.2 molar Na-phosphate is added (pH 7.0). The conjugation mixture is applied to gel filtration column (e.g., G-25) to remove unreacted SP-TNB and eluted. The mixture was fixture purified by HPLC gel filtration to remove unreacted PE polypeptide. Fractions are collected and analyzed by Western blot to confirm the presence of Pseudomonas exotoxin domain (by reaction with monoclonal anti-PE antibody) and monoclonal anti-SP antibody (directed to the amidated carboxy terminus of the SP). The presence of a disulfide linkage is confirmed by reduction (e.g., with 2-ME or dithiothreito) followed by gel electrophoresis (e.g., SDS-PAGE) and Western blot analysis with the above referenced monoclonal antibodies. Fractions containing one to one (1:1) PE to SP molar relationship conjugates are retained and stored, e.g., at -80.degree. C.

Detailed Description Text (99):
Substance P-Pseudomonas Exotoxin Disulfide Linked Conjugate Selectively and Potently Kills Neurokinin-1 Receptor Expressing Cells

Detailed Description Text (111):
SP-Pseudomonas Exotoxin Disulfide Linked Conjugate Kills NK-1-Expressing Cells in Rat Spinal Column Dorsal Horn Cells In Vivo

Detailed Description Text (117):
SP-Pseudomonas Exotoxin Disulfide Linked Conjugate Administered In Vivo Decreased Pain Sensation in a Heat Hyperalgesia Test

Detailed Description Text (143):
SP-Pseudomonas Exotoxin Disulfide Linked Conjugate Administered To Patients with Chronic Pain

Detailed Description Paragraph Table (4):
SEQUENCE LISTING <100> GENERAL INFORMATION: <160> NUMBER OF SEQ ID NOS: 6 <200> SEQUENCE CHARACTERISTICS: <210> SEQ ID NO 1 <211> LENGTH: 85 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: Description of Artificial Sequenceexemplary Pseudomonas exotoxin translocation domain (domain

II), residues 280-364 of SEQ ID NO6, except initiating Met in place of Gly at residue 280 <400> SEQUENCE: 1 Met Trp Glu Gln Leu Glu Gln Cys Gly Tyr Pro Val Gln Arg Leu Val 1 5 10 15 Ala Leu Tyr Leu Ala Ala Arg Leu Ser Trp Asn Gln Val Asp Gln Val 20 25 30 Ile Arg Asn Ala Leu Ala Ser Pro Gly Ser Gly Gly Asp Leu Gly Glu 35 40 45 Ala Ile Arg Glu Gln Pro Glu Gln Ala Arg Leu Ala Leu Thr Leu Ala 50 55 60 Ala Ala Glu Ser Glu Arg Phe Val Arg Gln Gly Thr Gly Asn Asp Glu 65 70 75 80 Ala Gly Ala Ala Asn 85 <200> SEQUENCE CHARACTERISTICS: <210> SEQ ID NO 2 <211> LENGTH: 214 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: Description of Artificial Sequenceexemplary Pseudomonas exotoxin ADP-ribosylation domain (domain III), residues 400-613 of SEQ ID NO6 <400> SEQUENCE: 2 Phe Leu Gly Asp Gly Gly Asp Val Ser Phe Ser Thr Arg Gly Thr Gln 1 5 10 15 Asn Trp Thr Val Glu Arg Leu Leu Gln Ala His Arg Gln Leu Glu Glu 20 25 30 Arg Gly Tyr Val Phe Val Gly Tyr His Gly Thr Phe Leu Glu Ala Ala 35 40 45 Gln Ser Ile Val Phe Gly Gly Val Arg Ala Arg Ser Gln Asp Leu Asp 50 55 60 Ala Ile Trp Arg Gly Phe Tyr Ile Ala Gly Asp Pro Ala Leu Ala Tyr 65 70 75 80 Gly Tyr Ala Gln Asp Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn 85 90 95 Gly Ala Leu Leu Arg Val Tyr Val Pro Arg Ser Ser Leu Pro Gly Phe 100 105 110 Tyr Arg Thr Ser Leu Thr Leu Ala Ala Pro Glu Ala Ala Gly Glu Val 115 120 125 Glu Arg Leu Ile Gly His Pro Leu Pro Leu Arg Leu Asp Ala Ile Thr 130 135 140 Gly Pro Glu Glu Glu Gly Gly Arg Leu Glu Thr Ile Leu Gly Trp Pro 145 150 155 160 Leu Ala Glu Arg Thr Val Val Ile Pro Ser Ala Ile Pro Thr Asp Pro 165 170 175 Arg Asn Val Gly Gly Asp Leu Asp Pro Ser Ser Ile Pro Asp Lys Glu 180 185 190 Gln Ala Ile Ser Ala Leu Pro Asp Tyr Ala Ser Gln Pro Gly Lys Pro 195 200 205 Pro Arg Glu Asp Leu Lys 210 <200> SEQUENCE CHARACTERISTICS: <210> SEQ ID NO 3 <211> LENGTH: 11 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: Description of Artificial Sequencehuman Substance P (SP) <400> SEQUENCE: 3 Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met 1 5 10 <200> SEQUENCE CHARACTERISTICS: <210> SEQ ID NO 4 <211> LENGTH: 12 <212> TYPE: PRT <213> ORGANISM: Artificial Sequence <220> FEATURE: <223> OTHER INFORMATION: Description of Artificial Sequencemodified SP peptide with Cys added to amino terminus <400> SEQUENCE: 4 Cys Arg Pro Lys Pro Gln Gln Phe Phe Gly Leu Met 1 5 10 <200> SEQUENCE CHARACTERISTICS: <210> SEQ ID NO 5 <211> LENGTH: 1839 <212> TYPE: DNA <213> ORGANISM: Pseudomonas aeruginosa <220> FEATURE: <221> NAME/KEY: CDS <222> LOCATION: (1)..(1839) <223> OTHER INFORMATION: exotoxin A <400> SEQUENCE: 5 gcc gaa gaa gct ttc gac ctc tgg aac gaa tgc gcc aaa gcc tgc gtg 48 Ala Glu Glu Ala Phe Asp Leu Trp Asn Glu Cys Ala Lys Ala Cys Val 1 5 10 15 ctc gac ctc aag gac ggc gtg cgt tcc agc cgc atg agc gtc gac ccg 96 Leu Asp Leu Lys Asp Gly Val Arg Ser Ser Arg Met Ser Val Asp Pro 20 25 30 gcc atc gcc gac acc aac ggc cag ggc gtg ctg cac tac tcc atg gtc 144 Ala Ile Ala Asp Thr Asn Gly Gln Gly Val Leu His Tyr Ser Met Val 35 40 45 ctg gag ggc ggc aac gac gcg ctc aag ctg gcc atc gac aac gcc ctc 192 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn Ala Leu 50 55 60 agc atc acc agc gac ggc ctg acc atc cgc ctc gaa ggc ggc gtc gag 240 Ser Ile Thr Ser Asp Gly Leu Thr Ile Arg Leu Glu Gly Gly Val Glu 65 70 75 80 ccg aac aag ccg gtg cgc tac agc tac acg cgc cag gcg cgc ggc agt 288 Pro Asn Lys Pro Val Arg Tyr Ser Tyr Thr Arg Gln Ala Arg Gly Ser 85 90 95 tgg tcg ctg aac tgg ctg gta ccg atc ggc cac gag aag ccc tcg aac 336 Trp Ser Leu Asn Trp Leu Val Pro Ile Gly His Glu Lys Pro Ser Asn 100 105 110 atc aag gtg ttc atc cac gaa ctg aac gcc ggc aac cag ctc agc cac 384 Ile Lys Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 atg tcg ccg atc tac acc atc gag atg ggc gac gag ttg ctg gcg aag 432 Met Ser Pro Ile Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 ctg gcg cgc gat gcc acc ttc ttc gtc agg gcg cac gag agc aac gag 480 Leu Ala Arg Asp Ala Thr Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 atg cag ccg acg ctc gcc atc agc cat gcc ggg gtc agc gtg gtc atg 528 Met Gln Pro Thr Leu Ala Ile Ser His Ala Gly Val Ser Val Val Met 165 170 175 gcc cag acc cag ccg cgc cgg gaa aag cgc tgg agc gaa tgg gcc agc 576 Ala Gln Thr Gln Pro Arg Arg Glu Lys Arg Trp Ser Glu Trp Ala Ser 180 185 190 ggc aag gtg ttg tgc ctg ctc gac ccg ctg gac ggg gtc tac aac tac 624 Gly Lys Val Leu Cys Leu Leu Asp Pro Leu Asp Gly Val Tyr Asn Tyr 195 200 205 ctc gcc cag caa cgc tgc aac ctc gac gat acc tgg gaa ggc aag atc 672 Leu Ala Gln Gln Arg Cys Asn Leu Asp Asp Thr Trp Glu Gly Lys Ile 210 215 220 tac cgg gtg ctc gcc ggc aac ccg gcg aag cat gac ctg gac atc aaa 720 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile Lys 225 230 235 240 ccc acg gtc atc agt cat cgc ctg cac ttt ccc gag ggc ggc agc ctg 768 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu

Gly Gly Ser Leu 245 250 255 gcc gcg ctg acc gcg cac cag gct tgc cac ctg ccg ctg gag
act ttc 816 Ala Ala Leu Thr Ala His Gln Ala Cys His Leu Pro Leu Glu Thr Phe 260 265
270 acc cgt cat cgc cag ccg cgc ggc tgg gaa caa ctg gag cag tgc ggc 864 Thr Arg His
Arg Gln Pro Arg Gly Trp Glu Gln Leu Glu Gln Cys Gly 275 280 285 tat ccg gtg cag cgg
ctg gtc gcc ctc tac ctg gcg gcg cgg ctg tcg 912 Tyr Pro Val Gln Arg Leu Val Ala Leu
Tyr Leu Ala Ala Arg Leu Ser 290 295 300 tgg aac cag gtc gac cag gtg atc cgc aac gcc
ctg gcc agc ccc ggc 960 Trp Asn Gln Val Asp Gln Val Ile Arg Asn Ala Leu Ala Ser Pro
Gly 305 310 315 320 agc ggc ggc gac ctg ggc gaa gcg atc cgc gag cag ccg gag cag gcc
1008 Ser Gly Gly Asp Leu Gly Glu Ala Ile Arg Glu Gln Pro Glu Gln Ala 325 330 335
cgt ctg gcc ctg acc ctg gcc gcc gcc gag agc gag cgc ttc gtc cgg 1056 Arg Leu Ala
Leu Thr Leu Ala Ala Ala Glu Ser Glu Arg Phe Val Arg 340 345 350 cag ggc acc ggc aac
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Gly Ala Ala Asn Ala Asp Val Val 355 360 365 agc ctg acc tgc ccg gtc gcc gcc ggt gaa
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395 400 ctc ggc gac ggc ggc gac gtc agc ttc agc acc cgc ggc acg cag aac 1248 Leu
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Leu Gln Ala His Arg Gln Leu Glu Glu Arg 420 425 430 ggc tat gtg ttc gtc ggc tac cac
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Gln Glu Pro Asp Ala Arg Gly Arg Ile Arg Asn Gly 485 490 495 gcc ctg ctg cgg gtc tat
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Tyr Ser Met Val 35 40 45 Leu Glu Gly Gly Asn Asp Ala Leu Lys Leu Ala Ile Asp Asn
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Val Phe Ile His Glu Leu Asn Ala Gly Asn Gln Leu Ser His 115 120 125 Met Ser Pro Ile
Tyr Thr Ile Glu Met Gly Asp Glu Leu Leu Ala Lys 130 135 140 Leu Ala Arg Asp Ala Thr
Phe Phe Val Arg Ala His Glu Ser Asn Glu 145 150 155 160 Met Gln Pro Thr Leu Ala Ile
Ser His Ala Gly Val Ser Val Val Met 165 170 175 Ala Gln Thr Gln Pro Arg Arg Glu Lys
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Gly Lys Ile 210 215 220 Tyr Arg Val Leu Ala Gly Asn Pro Ala Lys His Asp Leu Asp Ile
Lys 225 230 235 240 Pro Thr Val Ile Ser His Arg Leu His Phe Pro Glu Gly Gly Ser Leu

Other Reference Publication (7):
FitzGerald, David J., "Construction of Immunotoxins Using Pseudomonas Exotoxin A,"

the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

9. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located within ten amino acid residues of the translocation domain amino terminus.

10. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located at the translocation domain amino terminus.

11. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group is a cysteine residue.

12. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain is covalently linked to the Pseudomonas exotoxin ADP-ribosylation domain.

13. The pharamceutical composition of claim 12, wherein the covalent linkage between the Pseudomonas exotoxin translocation domain and the Pseudomonas exotoxin ADP-ribosylation domain is a peptide bond.

14. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain comprises an amino acid sequence as set forth in SEQ ID NO:1 and the Pseudomonas exotoxin ADP-ribosylation domain comprises an amino acid sequence as set forth in SEQ ID NO:2.

16. A method for ablating an NK1 receptor expressing cell in a patient comprising administering to said patient a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to ablate an NK1 receptor expressing cell, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

18. A method of treating chronic pain without significantly afftecting basal nociceptive responses comprising administering to a subject in need thereof a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to treat chronic pain without significantly afftecting basal nociceptive responses, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

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Terms	Documents
L4 and (cys or cysteine or loop or disulfide or di-sulfide).ti,ab,clm.	9

Display Format: KWIC Change Format

Previous Page Next Page Go to Doc#

DOCUMENT-IDENTIFIER: US 5976877 A
TITLE: Proteins produced by human lymphocytes DNA sequence encoding these proteins and their pharmaceutical and biological uses

Detailed Description Text (77):
This extra loop acts as immunogen since it is probably exposed at the outside of the molecule and consequently is exposed to recognition by antibodies.

Previous Doc Next Doc Go to

L10: Entry 48 of 79 File: USPT Aug 31, 2004

DOCUMENT-IDENTIFIER: US 6783761 B2
** See image for Certificate of Correction **
TITLE: Chimeric peptide immunogens

Other Reference Publication (4):
Susan F. Kobs-Conrad, Ann Marie DiGeorge, Hyosil Lee and Pravin T.P. Kaumaya "Folding and Immunogenicity of a Loop-Structured Peptide Using the Zinc-Finger Motif" in Peptides 1992 C.H. Schneider And A.N. Eberle (Eds.) 1993 ESCOM Science Publisher BV.

L9: Entry 34 of 34 File: USPT Mar 2, 1993

US-PAT-NO: 5190873
DOCUMENT-IDENTIFIER: US 5190873 A

TITLE: Hybrid tryptophan aporepressor containing ligand binding sites

DATE-ISSUED: March 2, 1993

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Lernhardt; Waldemar	Solana Beach	CA		
Bourdon; Mario	San Diego	CA		
Youderian; Phil	Ramona	CA		

ASSIGNEE-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY	TYPE CODE
California Institute of Biological Research	La Jolla	CA			02

APPL-NO: 07/ 720222 [PALM]
DATE FILED: June 21, 1991

INT-CL: [05] C07K 13/00, C07K 17/00, C07K 17/02, C12P 21/00

US-CL-ISSUED: 435/177; 435/69.7, 435/69.1, 530/350, 530/812, 930/250
US-CL-CURRENT: 435/177; 435/69.1, 435/69.7, 530/350, 530/812, 930/250

FIELD-OF-SEARCH: 435/91, 435/69.7, 435/69.1, 435/177, 530/350, 530/812, 930/250

PRIOR-ART-DISCLOSED:

U.S. PATENT DOCUMENTS

Search Selected Search ALL Clear

	PAT-NO	ISSUE-DATE	PATENTEE-NAME	US-CL
☐	4703039	October 1987	Hawiger et al.	514/21

FOREIGN PATENT DOCUMENTS

FOREIGN-PAT-NO	PUBN-DATE	COUNTRY	US-CL
9100912	January 1991	WO	

OTHER PUBLICATIONS

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Bass, et al. Science 242:240-245 1988.
Gunsalus, et al. PNAS 77(12) 7117-7121 1980.
Joachimiak et al., J. Biol. Chem., 258:12641-12643 (1983).
Lawson et al., Nature, 333:869-871 (1988).
Luisi et al., Biochim. Biophys. Acta, 1048:113-126 (1990).
Marmorstein et al., J. Biol. Chem., 262:4922-4927 (1987).
Pfau et al., Nucl. Acids Res., 18:6165 (1990).
Schevitz et al., Nature, 317:782-786 (1985).
Arrowsmith et al., Biochem., 29:6332-6341 (1990).
Arvidson et al., J. Biol. Chem., 261:238-243 (1986).
Arvidson et al., Genetics, 128:29-35 (1991).
Bourdon et al., J. Cell Biol., 108:1149-1155 (1989).
Joachimiak et al., Proc. Natl. Acad. Sci. USA, 80:668-672 (1983).
Zhang et al., Nature, 327:591-597 (1987).
Engel et al., Biochem. 30: 3161-3169 (1991).

ART-UNIT: 186

PRIMARY-EXAMINER: Lacey; David L.

ASSISTANT-EXAMINER: Adams; Donald E.

ATTY-AGENT-FIRM: Fitting; Thomas

ABSTRACT:

Hybrid proteins containing repressor proteins and substituted receptor binding sites, amino acid and DNA sequences encoding the hybrid proteins are provided. Methods for preparing the hybrid proteins are also described.

19 Claims, 6 Drawing figures
Exemplary Claim Number: 1
Number of Drawing Sheets: 6

GOVT-INTEREST:

This invention was made with government support under government contract 5 29 CA45506 awarded by the National Institutes of Health. The government has certain rights in the invention.

BRIEF SUMMARY:

1 FIELD OF THE INVENTION

2 This invention relates to hybrid proteins constructed from prokaryotic repressor proteins and receptor binding sites, and to the methods for producing the hybrid proteins.

3 BACKGROUND OF THE INVENTION

4 Receptors are molecules, typically proteins or glycoproteins, found on the surface of cells, including mammalian cells, that possess specific affinity

for other molecules known as ligands. Ligands may be small or large (macro) molecules such as proteins. Binding of ligands to receptors on the surface of mammalian cells elicits dramatic responses or "signals" in the cells such as proliferation and adhesion. These cellular responses involve protein-protein interactions and intercellular interactions that regulate important physiological processes, such as the humoral immune response. Defects in receptor structure and function may interfere with recognition of ligands by the cell-bound receptors resulting in disease or dysfunction and death.

5 Many cell surface receptors and their corresponding ligands have been identified and characterized structurally and biochemically. The study of protein-mediated intercellular signalling yields the surprising result that only short stretches of amino acids, e.g. at least three amino acids, on the surfaces of mammalian proteins are both necessary and sufficient to bind specific receptors and thereby elicit dramatic cellular responses.

6 Examples of characterized receptors include complement receptor type 2 (CD21 or "CR2") (Weigle et al., In Complement, Muller-Eberhard and Miescher, Eds., Springer-Verlag, Berlin, p. 323 (1985)). The clonal expansion of mature, antigen-reactive B lymphocytes in the humoral immune response is regulated both by direct intercellular interactions (with T helper lymphocytes and accessory cells), and by interactions with soluble growth factors (Unanue, Adv. Immunol., 15:95 (1972)). These soluble factors include B cell growth factors, interleukins, interferons, and components of the complement system (O'Garra et al., Immunol. Today, 8:45 (1988); Weigle et al., supra). Members of the latter two classes of proteins bind specific cell surface receptors, including CR2. CR2 is the B lymphocyte receptor for the proteolytic activation products C3bi, C3dg and C3d of complement component C3 (Cooper et al., Ann. Rev. Immunol., 6:85 (1988); Aggregated C3b and C3d induce B cell proliferation (Erdei et al., Eur. J. Immunol., 15:184 (1985); Melchers et al., Nature, 317:264 (1985)). CR2 is also the receptor for Epstein-Barr virus (EBV), a potent polyclonal B cell activator (Fingeroth et al., Proc. Natl. Acad. Sci. USA, 81:4510 (1985); Frade et al., Proc. Natl. Acad. Sci. USA, 82:1490 (1985)). CR2 plays a central role in signalling B cell proliferation (Cooper et al., supra (1988)). Several monoclonal antibodies and polyclonal anti-CR2 antisera stimulate T cell-dependent B cell proliferation (Cooper et al., supra (1988)). Furthermore, ligand binding to CR2 is necessary for the transition from G.sub.1 to S phase of the B cell cycle of human and murine preactivated blasts (Melchers, supra (1985); Bohnsack and Cooper J. Immunol., 141:2569 (1988)). CR2 is also phosphorylated during B cell stimulation, a common property of growth factor receptors (Changelian and Fearon J. Exp. Med., 161:101 (1986)).

7 CR2 occurs on normal and malignant B lymphocytes (Cooper et al., supra (1988); Hatzfeld et al , J. Immunol., 140:170 (1988)), on epithelial cells (Young et al., The Lancet, 240 (1986)), and, to a lesser extent, on immature thymocytes and follicular dendritic cells (Tsoukas and Lambris, Eru. J. Immunol., 18:1299 (1988); Reynes et al., J. Immunol., 135:2687 (1985)). The primary structure of CR2 has been deduced from the DNA sequence of its clone. Human CR2 is a membrane glycoprotein of 145 kd, and has sequence similarity to other members of the family of complement binding proteins.

8 The recognition sites on CR2 for C3d and EBV have been located on the N-terminal part of this longitudinal molecule. The sequence motifs on C3 and EBV coat protein, gp350, that mediate binding to CR2 receptor have also been defined (Lambris et al., Proc. Natl. Acad. Sci. USA, 82:4235 (1985); Nemerow et al., Cell 56:369 (1989)) (Table 1). Synthetic hexapeptides with the sequence of the CR2 binding site on C3 inhibit human and murine B cell proliferation (Lernhardt et al., Immunol. Rev., 99:239 (1987)). Thus CR2

receptor can bind both monomeric C3d and aggregated C3d as ligands, as well as the major epitope of EBV capsid protein.

9 CR2 ligands act in concert with other B cell growth modulators, including growth factors, lymphokines, and cytokines. Thus, the growth-inducing effect of anti-CR2 monoclonal antibody OKB7 is T cell-dependent, and requires T cell-derived B cell growth factors (Cooper et al., supra (1988)). It has been shown that optimal cell cycle progression and cell division occurs only in the presence of both anti-Ig antibodies and IL-2 or IL-5.

10 The CR2 receptor is of clinical interest, because it is the receptor for Epstein-Barr virus (EBV) (Frade supra (1985)). EBV is the causative agent of infectious mononucleosis (Huang et al., Int. J. Cancer 14:580 (1974)), and possibly is a human cancer virus, because its presence is correlated with nasopharyngeal carcinoma and Burkitt's lymphoma (Henle et al., Science, 157:1064 (1967)). In addition, EBV may play a role in the onset of B cell neoplasia observed in a substantial fraction of AIDS patients (Yarchoan et al., J. Clin. Invest., 78:439 (1986)). At the least, a substantial fraction of AIDS patients have chronic EBV infections. Exposure of pregnant women to individuals infected with and shedding EBV poses a significant risk to fetal development. It would be useful to better understand the mechanism of CR2 ligand action and to design and engineer proteins that function as recombinant inhibitors of EBV infection and lymphoma proliferation.

11 Prokaryotic repressors are small, multimeric proteins that are easy to manipulate genetically. Prokaryotic repressors bind short stretches of DNA called operators. Aporepressor proteins bind operators poorly and must complex with other small molecules called corepressors, such as tryptophan or S-adenosylmethionine to form active repressor complexes, or simply, repressors. Corepressors act as "keystones" that fit into and stabilize the hydrophobic cores of their aporepressors.

12 The E. Coli Tryptophan (Trp) aporepressor monomer is a peptide 108 amino acids long (M.sub.r =12,356 daltons) (Gunsalus and Yanofsky, Proc. Nat. Acad. Sci. USA, 77:7117-7121 (1980)) that assembles as a dimer (Joachimiak et al., Proc. Natl. Acad. Sci. USA, 80:668-672 (1983); Arvidson et al., J. Biol. Chem., 261:238-243 (1986)). Trp aporepressor binds DNA poorly in the absence of the corepressor ligand, L-tryptophan (or the analog 5-methyltryptophan, 5-MT). Aporepressor assembles with tryptophan or 5-MT to form active Trp repressor complex, a global repressor that binds operator sites to regulate the initiation of transcription from at least three different E. coli promoters. In addition, aporepressor can form inactive Trp pseudorepressor complexes with indole-3-propionic acid (IPA) or indole-.pi.-acrylic acid (IAA); these pseudo-repressor complexes bind operator DNA more poorly than aporepressor (Doolittle and Yanofsky, J. Bacteriol., 95:1283-1294 (1968); Baker and Yanofsky, Proc. Natl. Acad. Sci USA, 60:313-320 (1968)).

13 Trp aporepressor controls three operons that comprise a system to maintain the concentration of L-tryptophan in E. coli homeostatically, within levels necessary for efficient protein synthesis. When concentrations of intracellular tryptophan are low, TrpR exists predominantly as an aporepressor that cannot bind trp operator DNA, and the trpEDCBA biosynthetic genes are expressed maximally. When L-tryptophan levels are high, a substantial fraction of TrpR is active repressor, and tryptophan biosynthesis slows (Cohen and Jacob, C.R. Acad. Sci. Paris, 248:3490-3492 (1959); Yanofsky, J. Amer. Med. Assoc., 218:1026-1035 (1971); Bennet et al., Proc. Natl. Acad. Sci. USA, 73:2351-2355 (1976); Zurawski et al., J. Mol. Biol., 145:47-73 (1981); Yanofsky et al., J. Bacteriol., 158: 1018-1024 (1984)). Trp aporepressor regulates a biosynthetic pathway in response to the amount of an end product;

thus, it functions as a rheostat, rather than an on/off switch. In contrast, .mu. and other phage repressors control binary developmental decisions, and are not known to respond to small ligands. Other ligand-activated DNA-binding proteins have been studied to lesser extents.

14 The X-ray crystal structures of two forms of Trp repressor (Schevitz et al., Nature, 317:782-786 (1985); Lawson et al., Proteins, 3:18-31 (1988)), aporepressor (Zhang et al., Nature, 327:591-597 (1987)), and pseudorepressor (Lawson et al., Nature, 333:869-871 (1988)) have been determined, and show that, when crystallized, the peptide monomer is a bundle of six .alpha.-helices with a disordered, 11-residue N-terminal arm. The TrpR dimer has a remarkable subunit interface, in which four of each subunit's six .alpha.-interface, helices (A, B, C, and F) are interlocked. The amino acid sequence of the two flexible .alpha.-helices, D and E, resembles the conserved "helix-turn-helix" DNA-binding motif characteristic of many prokaryotic repressors, and pairs of the 2.degree. substructures formed by the D loop and E are positioned on the surface of Trp repressor to contact successive major grooves of trp operator DNA. Genetic analyses of mutant TrpR genes show that residues from both D and E are critical for DNA-binding (Bass et al., Science, 242:240-245 (1988); Kelly et al., Proc. Natl. Acad. Sci USA, 79:3120-3124 (1982)).

15 Recently, Arrowsmith, Jardetsky and coworkers have determined the structure of Trp repressor in solution, using .sup.1 H-NMR spectroscopic methods (Arrowsmith et al., Biochemistry, 29:6332 (1990); Arrowsmith and Jardetsky, submitted for publication (1991). Their results show that the structure of Trp repressor in solution resembles the crystal structures closely, with two major differences. In solution, the first half of A is partially disordered, and the residues organized as D in the crystal do not form an .alpha.-helix, but rather comprise some sort of surface loop (the "D Loop") (Arrowsmith et al., supra (1990)). The binding of the corepressor, L-tryptophan, restricts the motion of the D loop; amide protons of residues in D become less solvent-accessible in the presence of corepressor. However, corepressor binding does not elicit a coil-to-helix transition, because these protons remain uninvolved in H-bond formation in the repressor complex and in the specific repressor/operator complex [Arrowsmith and Jardetsky, supra.

16 Trp aporepressor is usually stable, and may be purified in large quantities (Arvidson et al., In Protein Purification: Micro to Macro, UCLA Symp. Mol. Cell Biol (Ed. Burgess), Alan R. Liss, NY; (1986); Smith et al., Proc. Natl. Acad. Sci., USA. 82:6104-6108 (1985)). In addition, to understand how particular amino acids contribute to the structure and function of Trp repressor, methods have been developed for both mismatch-primer (Arvidson et al., Genetics, 128 (1991) and cassette-style (Pfau and Youderian, Nuc. Acids Res., 18:6165 (1990) mutagenesis of either single or multiple adjacent codons of TroR. Mutagenesis may be coupled with a rapid screen for Trp repressor function; this screen depends on the color of colonies made by a strain of bacteria, CG103, which overproduces Trp repressor.

17 Comparisons of the NMR structures of Trp aporepressor and repressor suggests that the binding of indole analogs results in subtle changes in the orientation of D and E relative to the stationary hydrophobic core of the protein. The TrpR dimer has two identical, independent binding sites for corepressor (Arvidson et al., supra (1986); Marmorstein et al., J. Biol. Chem., 262:4922-4927 (1987)); surprisingly, these are formed by the side chains of residues from both monomers in a dimer (Schevitz et al., supra (1985)). The interactions that each corepressor is predicted to make with aporepressor are primarily hydrophobic. Presumably, the binding of corepressor restricts the ensemble of preferred conformations of the DNA-binding domains of an aporepressor to a subset of conformations that bind DNA with lower free

energies (pseudorepressor binding restricts aporepressor conformations to a subset that binds DNA with higher free energies).

18 Attempts have been made to construct hybrids between structural proteins and receptor binding sites. For example, hybrids between proteins having highly repetitive sequences such as silk-like protein (SLP) and the ten-residue RGD motif of fibronectin have been described (Cappello and Crissman, Chemical and Engineering News, pp. 26-32 (July 16, 1990)). Although the hybrid protein is active in vitro, its highly repetitive gene is unstable.

19 It would be advantageous to provide a method for producing hybrid proteins containing receptor binding sites, that are active as ligands for mammalian cell receptors to design reagents for a variety of applications including treatment of diseases resulting from receptor/ligand dysfunction.

20 SUMMARY OF THE INVENTION

21 The present invention provides such method and hybrid proteins. The hybrid proteins are produced from a prokaryotic repressor protein and a peptide segment that is heterologous to (not naturally present in) the repressor. Preferably, the peptide segment defines a binding site from a ligand reactive with a mammalian cell surface receptor. Examples of prokaryotic repressor protein that may be used include TrpR aporepressor protein, MET aporepressor protein, bacteriophage lambda, Lac repressor, bacteriophage P22 Arc repressor and the like. The invention also provides DNA sequences encoding the amino acid sequences of the invention, vectors containing the DNA sequence and host cells transfected with the vectors.

22 The receptor binding site can be obtained from interferon alpha, fibrinogen gamma, tenascin, fibronectin, and the like. Viral immunogenic pathogen derived proteins, such as proteins from HIV, EBV, hepatitis B and the like can also be used.

23 The invention also provides a method for preparing a hybrid protein prokaryotic repressor protein containing at least one substituted binding site from a ligand reactive with an eukaryotic cell surface receptor by 1) mutating the DNA codons for at least one selected amino acid in the amino acid sequence encoding the prokaryotic repressor to encode a contiguous sequence of amino acids that encodes a selected receptor binding site from a ligand reactive with an eukaryotic cell receptor to produce a mutated amino acid sequence; 2 a hybrid protein encoded by the mutated DNA sequence that has the activity of the selected receptor binding site; and isolating the hybrid protein produced. The hybrid protein is then purified and tested for biological activity as a binding site for eukaryotic cell receptors. The hybrid protein is produced by recombinant means or by chemical synthesis. A preferred prokaryotic repressor protein is Trp aporepressor.

DRAWING DESCRIPTION:

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a diagram illustrating the Construction of pTrpR-TN Plasmids. Single stranded DNA template was prepared by M13 infection of CJ236 transformed with pPY150. Site direct mutagenesis was done on single stranded DNA with oligonucleotides (38-45 mer) and transformation into CG103F.Kan. Tac P/O tac promotor/operator fragment rpoc+: rpoC transcription terminator.

FIG. 2 illustrates cell Attachment to TrpR-TN5. Shown is cell attachment (relative

fluorescence) of Di-I stained U251 human glioma cells to wells elated with increasing amounts of TrpR-TN5 as described in Example 7.

FIG. 3 illustrates cell Attachment to TrpR-TN5 and TrpR-TN1. Shown is cell attachment of U251 cells to TrpR-TN5, TrpRTN1 and TrpR in the presence (+) or absence 6(-) of 1mg/ml SRRGDMS peptide as described in Example 7.

FIG. 4 illustrates peptide Inhibition of Cell Attachment to Trp-TN5. Increasing concentrations of peptide SRRGDMS-NH.sub.2 and GRGDSP-NH.sub.2 were present in cell attachment assays on TrpR-TN5 as described in Example 7. The control peptide MGSRSRD did not inhibit cell attachment.

FIG. 5 illustrates .alpha..sub.v .beta..sub.3 -liposome binding to TrpR-TN5. Receptor liposomes were prepared by dialyses of receptor and phosphatidylcholine (PC) plus .sup.3 H-PC as described in Example 7. Shown is liposome binding to fibronectin (FN) vitronectin (VN), TrpR-TN5, and TrpR coated wells.

FIG. 6 illustrates inhibition of Cell Attachment by LM609
Anti .alpha..sub.v .beta..sub.3. Shown is cell attachment in media (or monoclonal antibody 81C6 1/200) and in presence of LM 609 1/200 as described in Example 7.

DETAILED DESCRIPTION:

1 DETAILED DESCRIPTION OF THE INVENTION

2 DEFINITIONS

3 Amino Acid Residue: An amino acid formed upon chemical digestion (hydrolysis) of a polypeptide at its peptide linkages. The amino acid residues described herein are preferably in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property is retained by the polypeptide. NH.sub.2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature (described in J. Biol. Chem., 243:3552-59 (1969) and adopted at 37 C.F.R. 1.822(b)(2)), abbreviations for amino acid residues are shown in the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL 1-Letter	3-Letter	AMINO ACID
Y	Tyr	tyrosine
G	Gly	glycine
F	Phe	phenylalanine
M	Met	methionine
A	Ala	alanine
S	Ser	serine
I	Ile	isoleucine
L	Leu	leucine
T	Thr	threonine
V	Val	valine
P	Pro	proline
K	Lys	lysine

H	His	histidine
Q	Gln	glutamine
E	Glu	glutamic acid
Z	Glx	Glu and/or Gln
W	Trp	tryptophan
R	Arg	arginine
D	Asp	aspartic acid
N	Asn	asparagine
B	Asx	Asn and/or Asp
C	Cys	cysteine
J	Xaa	Unknown or other

4 It should be noted that all amino acid residue sequences represented herein by formulae have a left-to-right orientation in the conventional direction of amino terminus to carboxy terminus. In addition, the phrase "amino acid residue" is broadly defined to include the amino acids listed in the Table of Correspondence and modified an unusual amino acids, such as those listed in 37 C.F. R. 1.822(b)(4), and incorporated herein by reference. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino acid residues or a covalent bond to an amino-terminal group such as NH.sub.2 or acetyl or to a carboxy-terminal group such as COOH.

5 Nucleotide: A monomeric unit of DNA or RNA consisting of a sugar moiety (pentose), a phosphate, and a nitrogenous heterocyclic base. The base is linked to the sugar moiety via the glycosidic carbon (1' carbon of the pentose) and that combination of base and sugar is a nucleoside. When the nucleoside contains a phosphate group bonded to the 3' or 5' position of the pentose it is referred to as a nucleotide. A sequence of operatively linked nucleotides is typically referred to herein as a "base sequence" or "nucleotide sequence", and their grammatical equivalents, and is represented herein by a formula whose left to right orientation is in the conventional direction of 5'-terminus to 3'-terminus.

6 Base Pair (bp): A partnership of adenine (A) with thymine (T), or of cytosine (C) with guanine (G) in a double stranded DNA molecule. In RNA, uracil (U) is substituted for thymine.

7 Nucleic Acid: A polymer of nucleotides, either single or double stranded.

8 Polynucleotide: a polymer of single or double stranded nucleotides. As used herein "polynucleotide" and its grammatical equivalents will include the full range of nucleic acids. A polynucleotide will typically refer to a nucleic acid molecule comprised of a linear strand of two or more deoxyribonucleotides and/or ribonucleotides. The exact size will depend on many factors, which in turn depends on the ultimate conditions of use, as is well known in the art. The polynucleotides of the present invention include primers, probes, RNA/DNA segments, oligonucleotides or "oligos" (relatively short polynucleotides), genes, vectors, plasmids, and the like.

9 Gene: A nucleic acid whose nucleotide sequence codes for an RNA or polypeptide. A gene can be either RNA or DNA.

10 Duplex DNA: a double-stranded nucleic acid molecule comprising two strands of substantially complementary polynucleotides held together by one or more hydrogen bonds between each of the complementary bases present in a base pair

of the duplex. Because the nucleotides that form a base pair can be either a ribonucleotide base or a deoxyribonucleotide base, the phrase "duplex DNA" refers to either a DNA-DNA duplex comprising two DNA strands (ds DNA), or an RNA-DNA duplex comprising one DNA and one RNA strand.

11 Complementary Bases: Nucleotides that normally pair up when DNA or RNA adopts a double stranded configuration.

12 Complementary Nucleotide Sequence: A sequence of nucleotides in a single-stranded molecule of DNA or RNA that is sufficiently complementary to that on another single strand to specifically hybridize to it with consequent hydrogen bonding.

13 Conserved: A nucleotide sequence is conserved with respect to a preselected (reference) sequence if it non-randomly hybridizes to an exact complement of the preselected sequence.

14 Hybridization: The pairing of substantially complementary nucleotide sequences (strands of nucleic acid) to form a duplex or heteroduplex by the establishment of hydrogen bonds between complementary base pairs. It is a specific, i.e. non-random, interaction between two complementary polynucleotides that ca be competitively inhibited.

15 Nucleotide Analog: A purine or pyrimidine nucleotide that differs structurally from A, T, G, C, or U, but is sufficiently similar to substitute for the normal nucleotide in a nucleic acid molecule.

16 DNA Homolog: Is a nucleic acid having a preselected conserved nucleotide sequence and a sequence coding for a receptor capable of binding a preselected ligand.

17 Recombinant DNA (rDNA) molecule: a DNA molecule produced by operatively linking two DNA segments. Thus, a recombinant DNA molecule is a hybrid DNA molecule comprising at least two nucleotide sequences not normally found together in nature. rDNA's not having a common biological origin, i.e., evolutionarily different, are said to be "heterologous".

18 Vector: a rDNA molecule capable of autonomous replication in a cell and to which a DNA segment, e.g., gene or polynucleotide, can be operatively linked so as to bring about replication of the attached segment. Vectors capable of directing the expression of genes encoding for one or more proteins are referred to herein as "expression vectors". Particularly important vectors allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

19 Receptor: A receptor is a molecule, such as a protein, glycoprotein and the like, that can specifically (non-randomly) bind to another molecule.

20 Antibody: The term antibody in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Exemplary antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and portions of an immunoglobulin molecule, including those portions known in the art as Fab, Fab', F(ab').sub.2 and F(v).

21 Antibody Combining Site: An antibody combining site is that structural portion

of an antibody molecule comprised of a heavy and light chain variable and hypervariable regions that specifically binds (immunoreacts with) an antigen. The term immunoreact in its various forms means specific binding between an antigenic determinant-containing molecule and a molecule containing an antibody combining site such as a whole antibody molecule or a portion thereof.

22 Monoclonal Antibody: The phrase monoclonal antibody in its various grammatical forms refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

23 Fusion Protein: A protein comprised of at least two polypeptides and a linking sequence to operatively link the two polypeptides into one continuous polypeptide. The two polypeptides linked in a fusion protein are typically derived from two independent sources, and therefore a fusion protein comprises two linked polypeptides not normally found linked in nature.

24 Upstream: In the direction opposite to the direction of DNA transcription, and therefore going from 5' to 3' on the non-coding strand, or 3' to 5' on the mRNA.

25 Downstream: Further along a DNA sequence in the direction of sequence transcription or read out, that is traveling in a 3'- to 5'-direction along the non-coding strand of the DNA or 5'- to 3'-direction along the RNA transcript.

26 Cistron: Sequence of nucleotides in a DNA molecule coding for an amino acid residue sequence and including upstream and downstream DNA expression control elements.

27 Stop Codon: Any of three codons that do not code for an amino acid, but instead cause termination of protein synthesis. They are UAG, UAA and UGA and are also referred to as a nonsense or termination codon.

28 The present invention concerns a method for preparing hybrid proteins consisting of prokaryotic repressors and receptor binding sites.

29 In the method of the invention, surface features of a stable, prokaryotic repressor, such as Trp aporepressor, are replaced with short peptide segments (oligopeptides) that are active as ligand binding sites for receptors produced by eukaryotic cells, such as antibodies, T-cell receptor, hormone receptors, and the like. The prokaryotic repressor acts as a scaffold, or "context" protein, for presentation of the peptide segment.

30 The peptide segment is typically at least 3, and preferably at least 5, and usually no more than about 20, and preferably no more than about 10, amino acid residues in length. Usually, the peptide segment will be in the range of 6 to 9 (inclusive) residues in length. The length must be sufficient for the segment to form a binding site such that the hybrid protein mimics at least one biological activity, as evidenced by binding, of the native protein ligand from which it is derived..

31 An exemplary hybrid protein is a tryptophan aporepressor containing within its amino acid residue sequence a peptide segment that is heterologous to (not naturally occurring within) the aporepressor. The peptide segment is located within the amino acid residue sequence of the hybrid protein such that it is expressed on the protein's solvent-accessible surface. Typically, the peptide sequence is flanked by amino-terminal and carboxy-terminal sequences corresponding to respective amino-and carboxy-terminal sequences of the aporepressor protein. However, the peptide segment can also be located at the amino- or carboxyterminus of the hybrid protein.

32 When the peptide segment is inserted or substituted between two flanking sequences, the amino acid residue sequence of the hybrid protein can be represented by the formula, read from left to right and in the direction of amino-terminus to carboxyterminus:

Z--B--U,

33 In the formula, Z is an amino-terminal portion of the tryptophan aporepressor, and U is a carboxyterminal portion of the tryptophan aporepressor. The lengths of Z and U are selected (adapted) such that B, the peptide segment, is expressed on the surface of the hybrid protein. Factors considered when adapting the length of Z and U are the length in residues of B, whether B is substituted or inserted without substitution into the aporepressor's native sequence, and in view of forming hybrid protein whose overall amino acid residue sequence length is about 100 to about 120 residues, preferably about 105 to about 115 residues, and more preferably about 108 to 112 residues. Typically, when the E.coli tryptophan aporepressor is used as the context protein, the hybrid protein length will be in the range of 108 residues because that is the length of the native aporepressor. For example, when a peptide segment that is 15 residues in length is to be inserted between flanking portions of the E.coli tryptophan aporepressor protein, about 15 residues should be deleted from the native aporepressor sequence to a hybrid protein length equivalent to that of the native aporepressor, i.e. about 108 residues. The residues to be deleted are those adjacent to the insertion site, i.e. interior residues, while the terminal portions, i.e. the amino- and carboxy- most (terminal) residues, are left intact. The dashes in the formula represent peptide bonds that link the flanking sequences to the peptide segment. Preferred hybrid proteins are represented by the amino acid residue sequences SEQ ID NO 1 through SEQ ID NO 19 listed below.

SEQ ID NO	
(1)	
	MAQQ-B- AAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPDEREALGT RVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVELRQW LEEVLLKSD
(2)	
	MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD EREALGTRVRIVEE-B- SQRELKNELGAGIATITRGSNSLKAAPVELRQWLEEVLLK SD
(3)	
	MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD EREALGTRVRIVEELLRGEM-B- ELGAGIATITRGSNSLKAAPVELRQWLEEVLLKSD

(4)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRELK-B-
ATITRGSNSLKAAPVELRQWLEEVLLKSD
(5)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRELKN-B-
ITRGSNSLKAAPVELRQWLEEVLLKSD
(6)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAA
PVELRQWLEEVLLKSD-B
(7)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQN-B-
LHLPLLNLMLTPDEREA
LGTRVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVEL
RQWLEEVLLKSD
(8)
MAQQ-B-
SPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPDERE
ALGTRVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVE
LRQWLEEVLLKSD
(9)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQ-B-
LHLPLLNLMLTPDEREAL
GTRVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVELR
QWLEEVLLKSD
(10)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELL-B-
QRELKNELGAGIATITRGSNSLKAAPVELRQWLEEVLLK SD
(11)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQ-B-
GIATITRGSNSLKAAPVELRQWLEEVLLKSD
(12)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRE-B-
ATITRGSNSLKAAPVELRQWLEEVLLKSD
(13)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAA
PVELRQWLEEVLL-B-
(14)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLH LPLLNLML-B-
TRVRI
VEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVELRQWLEEV
LLKSD
(15)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQR-B-
IATITRGSNSLKAAPVELRQWLEEVLLKSD
(16)
MAQQS-B-
AAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPDEREALGTR
VRIVEELLRGEMSQRELKNELGAGIATITRGSNSLKAAPVELRQWL
EEVLLKSD
(17)
MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEEL-B-

QRELKNELGAGIATITRGSNSLKAAPVELRQWLEEVLLK SD

(18)

MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMS-B-
ELGAGIATITRGSNSLKAAPVELRQWLEEVLLK SD

(19)

MAQQSPYSAAMAEQRHQEWLRFVDLLKNAYQNDLHLPLLNLMLTPD
EREALGTRVRIVEELLRGEMSQRELKN-B-
ATITRGSNSLKAAPVELRQWLEEVLLK SD

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34 When the peptide segment is located at the amino- or carboxy-terminus of the hybrid protein, the hybrid protein is represented by the formula B-U or Z-B, respectively, where Z and U are carboxy- or amino-terminal aporepressor sequences as previously described, and B is the peptide segment.

35 The phrases "surface accessible" and "surface expression" indicate that the peptide segment is positioned within the primary structure of the hybrid protein so that it is available for specific binding by a receptor when the hybrid is in a physiologically tolerable aqueous solution. Windows or zones which are available for surface expression or which are surface accessible in the E. coli tryptophan aporepressor are defined by residues 5-, 44-52, 61-67, 67-77, 71-81 and 108 of the native protein sequence as shown in SEQ ID NO 20.

36 In one embodiment of the invention, binding sites on the proteins interferon alpha (IFN) and gp350 (EBV coat protein) that interact with one major eukaryotic B lymphocyte surface receptor, CR2, are substituted or inserted as peptide segments into the E. coli Trp aporepressor protein to form novel hybrid proteins.

37 In another embodiment, the hybrid proteins, at the DNA level, are substrates for subsequent site-directed mutagenesis to generate mutant binding sites with altered biological properties, e.g. greater affinity for receptor, to provide additional hybrid proteins with novel activities.

38 Peptide segments forming binding sites can be substituted for or inserted into regions of the aporepressor regions whose conformation is restricted by corepressor binding, without disturbing the corepressor binding pocket. Corepressor analogs may be used to modify the conformations of the peptide segments. Thus, several different conformations of each peptide segment in the hybrid Trp aporepressor may be made with different resulting activities.

39 The method of the invention for preparing hybrid proteins is carried out as follows. Mutagenesis is performed on single or multiple codons of the prokaryotic repressor gene using a procedure such as that described by Kunkel (Proc. Natl. Acad. Sci. USA 82:488-492 (1985)) and, typically, an additional restriction selection step as described Wells, Philos. Trans. R. Soc. Lond. Ser. A 317:415-423 (1986)). In this procedure, the gene encoding the prokaryotic repressor, for example Trp aporepressor, is prepared, for example by introducing unique restriction endonuclease sites, and is inserted into an appropriate plasmid, such as pPY150, a rop.sup.+ derivative of pBR322, which expresses trpR from the lacUV5 promoter (Bass, Science 242:240-245 (1988)) or pTACTERM, a rop pBR322 derivative and site-directed mutagenesis to alter the sequence of the repressor to encode a desired peptide segment receptor binding site). This method can be used to make single changes, or to "randomize" a codon by replacing it with the mixed sequence NNS, in which N can be any of the four DNA nucleotides A, C, G or T and S can be C or G. This sequence

represents all 20 amino acids with 32 codons.

40 Substitutions are made in the prokaryotic repressor using site-directed mutagenesis as described above to alter the DNA sequence to replace a stretch of contiguous DNA in the sequence of the repressor with the sequence of at least one selected binding site. Preferably, the selected binding site substituted into the prokaryotic repressor comprises at least three, and preferably five amino acids. The gene encoding the repressor and substituted receptor binding site is then cloned and expressed using standard molecular biology procedures. The hybrid protein products are then purified to provide a hybrid repressor protein containing the substituted receptor binding site or sites.

41 Activity of the resulting hybrid protein is confirmed using a binding assay appropriate for the ligand being mimicked. Typically, this involves determining whether the protein binds t cells containing the receptor reactive with the binding site. Hybrid Trp repressor protein containing the tenascin receptor binding site Bourdon et al., J. Cell. Biol., 108:1149-1155 (1989) as described in Example 7, infra, an assay to determine whether the hybrid protein binds to human tumor cells is carried out using the all attachment assay described by Bourdon et al., supra.

42 The present invention also contemplates performing further mutagenesis in the receptor binding site sequences after prior mutagenesis of the repressor protein to provide the binding site sequence in the repressor protein to identify hybrid proteins with desired activities. Changes are made in the flanking codons for amino acid residues of the binding site in the hybrid protein to optimize the presentation of the site to its corresponding receptor. In addition, single amino acid changes are made in the binding site to optimize the primary sequence of the site. These will include mutations that increase the specific activity of the site as determined by standard binding assays.

43 Hybrid proteins produced by the methods of the invention may be used as reagents for treatment of or introduction into humans to combat infection or disease caused by defects in the interaction of the receptor and its ligands. Thus, binding sites for receptors from proteins involved in infection, for example from EBV virus proteins, may be engineered in hybrid proteins using the methods of the invention to combat EBV infection. Such proteins may mimic a compound such as interferon in vivo to block the binding or subsequent interactions of ligands to or with the receptors for EBV, preventing the effects of infection by this virus.

44 Therapeutic applications of the hybrid proteins of the invention are carried out using pharmaceutical compositions containing a pharmaceutically effective amount of the hybrid protein and a pharmaceutically acceptable carrier. The compositions may additionally include other reagents for treatment. Such compositions are administered using conventional modes of administration including, but not limited to, topical, intravenous, intraperitoneal, oral and intralymphatic introduction. The hybrid proteins are used in a variety of dosage forms which include, but are not limited to, liquid solutions or suspensions, tablets, pills, powders, suppositories, polymeric microcapsules or microvesicles, liposomes, and injectable or infusible solutions. The preferred form depends upon the mode of administration and the therapeutic application.

45 The compositions containing the hybrid proteins of the invention also preferably include conventional pharmaceutically acceptable carriers and

adjuvants known in the art such as human serum albumin, ion exchangers, alumina, lecithin, buffer substances such as phosphates, glycine, sorbic acid, potassium sorbate, and salts or electrolytes such as protamine sulfate.

46 The most effective mode of administration and dosage regimen for the compositions of the invention depends on the severity and course of the disease or dysfunctions, the patient's health and response to treatment, and the judgment of the treating physician. Accordingly, the dosages of the compositions are titrated to the individual patient.

47 Quantities of the hybrid proteins of the invention may be readily made and purified using additional standard procedures such as immunoaffinity, gel exclusion chromatography, ion exchange chromatography electrophoresis, and the like. Purified hybrid proteins may be used as vaccines to immunize against certain disease. Procedures for preparing such vaccines are known in the art (see, e.g. Estin et al., Proc. Natl. Acad. Sci. (USA) 85:1052 (1988)). Briefly, recombinant viruses are constructed for expression of the cloned gene encoding the hybrid protein. Cells infected with the recombinant viruses will express the hybrid protein at the surface of the cells together with the host's incompatibility antigens and immunogenic viral proteins. This favors the induction of cellular immunity which plays a key role in tumor rejection. A suitable virus, for example vaccinia virus derived from a plaque-purified virus of the Wyeth smallpox vaccine (New York City Board of Health strain), is used to construct a recombinant virus containing the coding sequence of the hybrid protein under control of the vaccinia virus "7.5K" promoter (Hue et al., J. Virol. 62:176-180 (1988)). The recombinant virus may then be administered intravenously as a vaccine to protect against infection.

48 In addition to Trp repressor protein, any prokaryotic repressor proteins may be used as the context protein for expressing, in a conformationally restricted manner, a receptor binding site. For example, the MET repressor, bacteriophage lambda repressor phage P22Arc repressor, and the like, can be used in place of the Trp repressor to carry out the invention.

49 Receptor binding sites for use in the present invention are likewise not limited to those shown in the examples herein, i.e. fibronectin, tenascin, or IFN . Any binding site that recognizes a cell surface receptor for which the amino acid or DNA sequence is known may be used to substitute or insert into the repressor protein to form a hybrid.

50 The invention described herein further comprises DNA sequences encoding the hybrid monomers previously described. As used herein, the term "DNA sequences" encompasses both double-stranded DNA and single-stranded DNA containing information equivalent to that of the amino acid sequences as determined by the genetic code; such single-stranded sequences can be either in the sense strand orientation or the antisense strand orientation.

51 A double-stranded DNA sequence encoding a hybrid monomer according to the present invention can be operatively linked to a transcription-effecting DNA sequence capable of effecting transcription of the DNA sequence encoding the monomer. This transcription-effecting sequence is preferably one of Escherichia coli Lac promoter, E. coli trp promoter, bacteriophage lambda P.sub.L promoter, and tac promoter, a hybrid trp-lac promoter, but other promoters are known in the art and can be used.

52 This DNA sequence, comprising a DNA sequence encoding a hybrid monomer operatively linked to a transcription-effecting DNA sequence, can be incorporated into a plasmid capable of stably transforming prokaryotic host

cells to form a vector. The plasmid preferably has both a drug resistance marker and a replication origin. Many suitable plasmids are known in the art, including, but not limited to, the following: pBR322 and its derivatives; pUC18, pUC19, and their derivatives; bacteriophage .mu.-derived plasmids, and bacteriophage M13-derived plasmids. Suitable host-vector systems are described, for example, in B. Perbal, "A Practical Guide to Molecular Cloning" (2d ed., John Wiley & Sons, New York, 1988), and "Guide to Molecular Cloning Techniques" (Berger & Kimmel, eds., Academic Press, New York, 1987; Volume 152 of Methods in Enzymology). Preferably, the prokaryotic host cells are E. coli. Prokaryotic, eukaryotic and archaebacterial host cells stably transformed with such a vector are also within the present invention.

53 EXAMPLES

54 The following description provides details of the manner in which particular embodiments of the present invention may be made and used. This description, while exemplary of the present invention, is not to be construed as specifically limiting the invention. Variations and equivalents, now known or later developed, which would be within the understanding and technical competence of one skilled in this art are to be considered as falling within the scope of this invention.

55 1. Oligonucleotide Design for Producing Trp Aporepressor Hybrid Recombinant DNA

56 In this invention, surface features of a stable procaryotic aporepressor, in this instance E. coli tryptophan aporepressor (TrpR), are replaced with short oligopeptides that are active as ligands or binding sites for receptors on eucaryotic cells. The procaryotic aporepressor then acts as a scaffold or context protein to allow presentation of the oligopeptide binding site. The nature of the conformation of a receptor binding site can then be evaluated with respect to the specificity of its interactions with its receptor.

57 To achieve this invention, different regions of the gene encoding TrpR were separately subjected to oligonucleotide-mediated site-directed mutagenesis as described below to produce receptor binding sites in those locations. The nucleotide sequence for a potential integrin binding site derived from human tenascin was engineered into eight different regions on the surface of TrpR. The regions were mutagenized such that a fusion protein having the amino acid residue sequence SRRGDMS (SEQ ID NO 21) flanked by TrpR amino acid residues was produced.

58 Multiple sites on the TrpR gene which is 324 base pairs in length were chosen to provide a reasonable opportunity to place the engineered recombinant binding site in a variety of structural contexts, one or more of which may be appropriate for receptor binding in the fusion protein. The rationale for selecting particular sites for site-directed mutagenesis was based on the extensive nuclear magnetic resonance (NMR) and X-ray crystallographic characterization of Trp aporepressor protein. See Joachimiak et al., J. Biol. Chem., 258:12641-12643 (1983); Luisi et al., Biochim. Biophys. Acta., 1048:113-126 (1990); and Arrowsmith et al., Biochem., 29:6332 (1990).

59 Briefly, E.coli Trp aporepressor is a small dimeric regulatory protein that binds two molecules of the corepressor, L-tryptophan, to form the active repressor complex. The aporepressor has been crystallized in more than eleven different forms, most of which diffract cl

4. Preparation of TrpR-Heterologous Receptor Binding Site Recombinants to Produce Cysteine Disulfide Loops

95 a. TrpR Recombinant Hybrids Having an Integrin Receptor Binding Site Containing Cysteine Disulfide Loops

96 The conformational context of the SRRGDMS (SEQ ID NO 21) or GRGDSP (SEQ ID NO 46) sequence within TrpR is further altered by creating cysteine disulfide bridges to create a loop containing the binding site sequence. The rationale for this is several fold. First, modeling predictions (Cachau et al., J. Mol. Res., 2:179-186 (1989)) and results with cyclic peptides (Pierschbacher et al., J. Biol. Chem., 262:17294-17298 (1987)) and a helical dimers (Engel et al., Bichem., 30:3161-3169 (1991)) indicate that the most active conformations are likely to be contained in a turn structures or loops. Second, the snake venom disintegrin proteins have the RGD site flanked by cysteines (Gould et al., P.S.E.B.M., 195:168-171 (1990)). Third, the active TrpR-TN5 hybrid protein produced in Example 6 is likely to reside within an extended or loop structure. Finally, cysteine disulfide bridges constrain the binding sequence in ways not possible through the TrpR context alone. By selecting the flanking distance from the binding sequence, loops of various sizes and varying conformation contexts are generated. The formation of disulfide bridges is analogous to the formation of artificial cyclic peptides. However, unlike cyclic peptides the NH.sub.2 and COOH protein context is not lost.

97 This strategy is most applicable to TrpR-hybrids at the NH.sub.2 and COOH-termini (TrpR-TN1, TrpR-TN7) and D/E transition (TrpR-TN4, TN5, and TN6), because these sites are flexible regions of Trp aporepressor and changes in these regions are least likely to disrupt the hydrophobic core. Core disruption would make NMR spectroscopy and X-ray crystallographic analysis exceedingly difficult. Because both TrpR-Tn1 (NH.sub.2 -terminal) and TrpR-TN5 (D/E) are both active receptor binding proteins, cysteine bridges in these mutants are generated first. Cysteine pairs flanking the SRRGDMS sequence are introduced into the TrpR-TN1 and TN5 by a single round of site-directed mutagenesis as described in Example 3 using paired oligonucleotides coding for a cysteines flanking the SRRGDMS substitution site. The oligonucleotides used for generating hybrid TrpR-SRRGDMS fusion proteins having disulfide loops are listed in Table 2 below.

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      Set  Items  Description
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? s
Terminal set to DLINK
? t s5/9/28 35 20 19 14 11 10 4
>>>Null command ignored
? t s5/9/28 35 20 19 14 11 10 4
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5/9/28 (Item 28 from file: 155)

12137046 PMID: 9437495

Diphtheria toxin as a molecular tool in the study of acidic fibroblast growth factor signalling.

Wiedlocha A
Department of Biochemistry, Norwegian Radium Hospital, Oslo.

Archivum immunologiae et therapiae experimentalis (POLAND) 1997, 45 (5-6) p391-409, ISSN 0004-069X Journal Code: 0114365
Publishing Model Print
Document type: Journal Article; Review; Review, Tutorial
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS

In eukaryotic cells proteins are translocated across a number of cellular membranes into various intracellular organelles such as the endoplasmatic **reticulum** , mitochondria, peroxisomes and chloroplasts. In all these cases the proteins are translocated away from the cytosol. However, certain proteins are also translocated in the opposite direction, from the exterior to the cytosol. Well established examples are some bacterial and plant protein toxins, that exert their effect in the cytosol. A common property of protein toxins with intracellular action is that they contain two functionally different moieties, in many cases consisting of two. **disulfide** -linked polypeptides. Relatively little is known about how these proteins cross the membrane. The **translocation** process is best understood in the case of diphtheria toxin, which binds to **cell** surface receptors, is then taken up by endocytosis and is subsequently translocated to the cytosol, where it inactivates elongation factor 2. Recently it has been recognized that diphtheria toxin as well as a few other protein toxins can be used to carry passenger peptides or proteins into cells (in addition to other usefull roles which the toxins have begun to play in understanding many cellular processes and in certain prophylactic and therapeutic purposes). Here, the approach of using diphtheria toxin as a **translocation** vehicle in the study of new aspects of signal transduction mechanisms activated by acidic fibroblast growth factor is discussed and the possibility that some proteins have distinct functions in more than one cellular compartment is considered. Finally, this article focuses on the role of the toxins as tools in **cell** biology and experimental medicine. (178 Refs.)

Descriptors: *Diphtheria Toxin--physiology--PH; *Fibroblast Growth Factor 1--physiology--PH; *Molecular Probes--physiology--PH; *Signal Transduction ; Animals; Cell Line; Diphtheria Toxin--metabolism--ME; Fibroblast Growth Factor 1--biosynthesis--BI; Fibroblast Growth Factor 1--metabolism--ME; Humans; Molecular Probes--metabolism--ME; Organ Specificity; Receptors, Fibroblast Growth Factor--biosynthesis--BI; Receptors, Fibroblast Growth Factor--metabolism--ME; Receptors, Fibroblast Growth Factor--physiology

--PH
CAS Registry No.: 0 (Diphtheria Toxin); 0 (Molecular Probes); 0 (Receptors, Fibroblast Growth Factor); 104781-85-3 (Fibroblast Growth Factor 1)
Record Date Created: 19980209
Record Date Completed: 19980209

5/9/35 (Item 35 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

11809775 PMID: 9120397
Targeting of HIV-1 antigens for rapid intracellular degradation enhances cytotoxic T lymphocyte (CTL) recognition and the induction of de novo CTL responses in vivo after immunization.
Tobery T W; Siliciano R F
Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, USA.
Journal of experimental medicine (UNITED STATES) Mar 3 1997, 185 (5) p909-20, ISSN 0022-1007 Journal Code: 2985109R
Contract/Grant No.: AI28108; AI; NIAID; AI37924; AI; NIAID
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS; AIDS/HIV
CD8+ cytotoxic T lymphocytes (CTLs) have the ability to recognize and eliminate virally infected cells before new virions are produced within that **cell** . Therefore, a rapid and vigorous CD8+ CTL response, induced by vaccination, can, in principle, prevent disseminated infection in vaccinated individuals who are exposed to the relevant virus. There has thus been interest in novel vaccine strategies that will enhance the induction of CD8+ CTLs. In this study, we have tested the hypothesis that targeting an antigen to undergo more efficient processing by the class I processing pathway will elicit a more vigorous CD8+ CTL response against that antigen. Targeting a type I transmembrane protein, the **HIV** -1 envelope (env) protein, for expression in the cytoplasm, rather than allowing its normal co-translational **translocation** into the endoplasmic **reticulum** , sensitized target cells expressing this mutant more rapidly for lysis by an env-specific CTL clone. Additionally, a greatly enhanced de novo env-specific CTL response was induced in vivo after immunization of mice with recombinant vaccinia vectors expressing the cytoplasmic env mutant. Similarly, targeting a cytoplasmic protein, **HIV** -1 nef, to undergo rapid cytoplasmic degradation induced a greatly enhanced de novo nef-specific CD8+ CTL response in vivo after immunization of mice with either recombinant vaccinia vectors or DNA expression plasmids expressing the degradation targeted nef mutant. The targeting of viral antigens for rapid cytoplasmic degradation represents a novel and highly effective vaccine strategy for the induction of enhanced de novo CTL responses in vivo.
Tags: Comparative Study; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.
Descriptors: *AIDS Vaccines--immunology--IM; *Antigen Presentation; *HIV Antigens--immunology--IM; *HIV-1--immunology--IM; *T-Lymphocytes, Cytotoxic --immunology--IM; Animals; Cell Compartmentation; Cytoplasm--metabolism--ME ; Gene Products, env--genetics--GE; Gene Products, env--immunology--IM; Gene Products, nef--genetics--GE; Gene Products, nef--immunology--IM; HIV

Antigens--metabolism--ME; HIV Infections--prevention and control--PC; Humans; Immunization; Mice; Mice, Inbred BALB C; Models, Immunological; Recombinant Fusion Proteins--immunology--IM; Ubiquitins--genetics--GE; Ubiquitins--metabolism--ME
CAS Registry No.: 0 (AIDS Vaccines); 0 (Gene Products, env); 0 (Gene Products, nef); 0 (HIV Antigens); 0 (Recombinant Fusion Proteins); 0 (Ubiquitins)
Record Date Created: 19970421
Record Date Completed: 19970421

5/9/20 (Item 20 from file: 135)
DIALOG(R)File 135:NewsRx Weekly Reports

0000020998 (THIS IS THE FULLTEXT)

Vaccine Therapy Targets Antigens for CTL Attack

DeNoon, Daniel J.

AIDS Weekly, May 26, 1997, p.9-10

DOCUMENT TYPE: Editor's Choice LANGUAGE: English
RECORD TYPE: FULLTEXT
AUDIENCE: Professional
WORD COUNT: 551

TEXT: A new type of vaccine vastly increases elimination of HIV infected cells by cytotoxic lymphocytes (CTLs).

The technique uses viral genes that have been altered so that the antigens they express are presented to the immune system far more efficiently than they are during normal virus replication. Such vaccines theoretically could be adapted to express altered antigens from other intracellular pathogens or tumor cells, and could be used to prevent or treat disease.

"A rapid and vigorous CD8+ CTL response, induced by vaccination, can, in principle, prevent disseminated infection in vaccinated individuals who are exposed to the relevant virus," suggested Timothy W. Tobery of Johns Hopkins University School of Medicine.

Tobery discussed the novel strategy in a presentation to the 9th Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS (NCVDG), held May 4-7, 1997, in Bethesda, Maryland.

The strategy seeks to improve elimination of virus-infected cells by CTL, the immune system's principal means of fighting intracellular pathogens.

"Stimulation of and recognition by antigen-specific CD8 + CTL is dependent on the level of processing of the antigen," Tobery explained.

When a virus replicates within a **cell** , that **cell** puts out a call for help. It breaks down excess viral proteins in its lysosome, sends them to the **cell** nucleus, and finally expresses them on major histocompatibility complex (MHC) receptors where they sensitize CTL to attack cells carrying the viral proteins. But this process is inefficient.

"The vast majority of viral protein goes into [making new] virus," Tobery said. "Only about 5 percent is processed, sent to the nucleus, and expressed on MHC receptors."

An increasing body of evidence suggests that CTL are crucial for control of **HIV** infection, but that most infected individuals fail to mount a strong enough CTL response to prevent progression to AIDS.

Tobery and colleague Robert F. Siliciano created a gene, dubbed ss-env , encoding the **HIV** envelope protein but lacking the signalling sequence which normally would permit its cotranslational **translocation** to the

cellular endoplasmic **reticulum** for processing into new virions. Expression of ss-env instead occurs in the cytoplasm, where it can be processed by the **cell** for MHC presentation but cannot participate in virus replication.

Cells expressing ss-env were killed much more quickly by an Env-specific CTL clone than cells expressing normal env .

Mice immunized with vaccinia virus carrying ss-env (vac ss-env) developed "a very strong primary Env-specific CTL response," Tobery said.

The researchers used a slightly different approach to inducing CTL responses to another **HIV** protein, the crucial regulatory protein Nef. In this approach, Tobery and Siliciano targeted Nef for rapid degradation in the cellular cytoplasm.

They accomplished this by creating a ubiquitin-Nef fusion construct (UbRNef) that expresses ubiquitin linked to Nef via an arginine molecule. When expressed in the **cell** , this construct is degraded rapidly by cellular ubiquitin hydrolases.

UbRNef was incorporated into DNA expression plasmids or vaccinia virus. Inoculation of mice with either of these vaccines, Tobery said, induced "greatly enhanced" de novo Nef-specific CD8 + CTL responses.

"The targeting of viral antigens for rapid cytoplasmic degradation represents a novel and highly effective vaccine strategy for the induction of enhanced de novo CTL responses in vivo," Tobery concluded. - by Daniel J. DeNoon, Senior Editor

DESCRIPTORS: news
SUBJECT HEADING: Conference Coverage (NCVDG)

5/9/19 (Item 19 from file: 34)

04644957 Genuine Article#: TZ983 Number of References: 132
Title: COMMON PRINCIPLES OF PROTEIN TRANSLOCATION ACROSS MEMBRANES
Author(s): SCHATZ G; DOBBERSTEIN B
Corporate Source: UNIV BASEL,BIOCTR,KLINGELBERGSTR 70/CH-4056 BASEL//SWITZERLAND/; UNIV HEIDELBERG,ZENTRUM MOLEK BIOL/D-69120 HEIDELBERG//GERMANY/
Journal: SCIENCE, 1996, V271, N5255 (MAR 15), P1519-1526
ISSN: 0036-8075
Language: ENGLISH Document Type: REVIEW
Geographic Location: SWITZERLAND; GERMANY
Subfile: SciSearch; CC PHYS--Current Contents, Physical, Chemical & Earth Sciences; CC LIFE--Current Contents, Life Sciences; CC AGRI--Current Contents, Agriculture, Biology & Environmental Sciences
Journal Subject Category: MULTIDISCIPLINARY SCIENCES
Abstract: Most major systems that transport proteins across a membrane share the following features: an amino-terminal transient signal sequence on the transported protein, a targeting system on the cis side of the membrane, a hetero-oligomeric transmembrane channel that is gated both across and within the plane of the membrane, a peripherally attached protein translocation motor that is powered by the hydrolysis of nucleoside triphosphate, and a protein folding system on the trans side of the membrane. These transport systems are divided into two families: export systems that export proteins out of the cytosol, and import systems that transport proteins into cytosol-like compartments.
Identifiers--KeyWords Plus: REQUIRES NUCLEOSIDE TRIPHOSPHATES; MITOCHONDRIAL INNER MEMBRANE; IMMUNOGLOBULIN LIGHT-CHAINS; SIGNAL

RECOGNITION PARTICLE; ESCHERICHIA-COLI; ENDOPLASMIC-RETICULUM; YEAST MITOCHONDRIA; INTERMEMBRANE SPACE; PRECURSOR PROTEINS; THYLAKOID MEMBRANE

Research Fronts: 94-1621 001 (MITOCHONDRIAL PROCESSING PEPTIDASE; PROTEIN IMPORT; PREPROTEINS OF CHLOROPLAST ENVELOPE INNER MEMBRANE CONTAIN TARGETING INFORMATION)

94-1770 001 (PROTEIN **DISULFIDE** BOND FORMATION; HUMAN PLACENTAL ALKALINE-PHOSPHATASE IN ESCHERICHIA-COLI; ACTIVE-SITE CYSTEINE RESIDUES OF DSBA)

94-2145 001 (HEAT-SHOCK PROTEINS; CHAPERONIN GROEL; HSP70 STRESS GENE)

94-8053 001 (PROTEIN **TRANSLOCATION** ACROSS THE ESCHERICHIA-COLI INNER MEMBRANE; SECRETION PATHWAY; SUBFRACTIONATED **CELL** -FREE EXPORT SYSTEM)

94-8078 001 (PROTEIN **TRANSLOCATION** ACROSS THE ENDOPLASMIC- **RETICULUM** MEMBRANE; SIGNAL RECOGNITION PARTICLE FUNCTIONS; NUCLEAR-ENVELOPE ION CHANNELS)

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5/9/14 (Item 14 from file: 155)

09827960 PMID: 1620130

Topology and functional domains of Sec63p, an endoplasmic reticulum membrane protein required for secretory protein translocation.

Feldheim D; Rothblatt J; Schekman R
Howard Hughes Medical Research Institute, Division of Biochemistry and Molecular Biology, University of California, Berkeley 94720.

Molecular and cellular biology (UNITED STATES) Jul 1992, 12 (7) p3288-96, ISSN 0270-7306 Journal Code: 8109087
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS

SEC63 encodes a protein required for secretory protein **translocation** into the endoplasmic **reticulum** (ER) of Saccharomyces cerevisiae (J. A. Rothblatt, R. J. Deshaies, S. L. Sanders, G. Daum, and R. Schekman, J. **Cell** Biol. 109:2641-2652, 1989). Antibody directed against a recombinant form of the protein detects a 73-kDa polypeptide which, by immunofluorescence microscopy, is localized to the nuclear envelope-ER network. **Cell** fractionation and protease protection experiments confirm the prediction that Sec63p is an integral membrane protein. A series of SEC63-SUC2 fusion genes was created to assess the topology of Sec63p within the ER membrane. The largest hybrid proteins are unglycosylated, suggesting

that the carboxyl terminus of Sec63p faces the cytosol. Invertase fusion to a **loop** in Sec63p that is flanked by two putative transmembrane domains produces an extensively glycosylated hybrid protein. This **loop**, which is homologous to the amino terminus of the Escherichia coli heat shock protein, DnaJ, is likely to face the ER lumen. By analogy to the interaction of the DnaJ and Hsp70-like DnaK proteins in E. coli, the DnaJ **loop** of Sec63p may recruit luminal Hsp70 (BiP/GRP78/Kar2p) to the **translocation** apparatus. Mutations in two highly conserved positions of the DnaJ **loop** and short deletions of the carboxyl terminus inactivate Sec63p activity. Sec63p associates with several other proteins, including Sec61p, a 31.5-kDa glycoprotein, and a 23-kDa protein, and together with these proteins may constitute part of the polypeptide **translocation** apparatus. A nonfunctional DnaJ domain mutant allele does not interfere with the formation of the Sec63p/Sec61p/gp31.5/p23 complex.

Descriptors: *Fungal Proteins--genetics--GE; *Fungal Proteins--secretion --SE; *Membrane Proteins--genetics--GE; *Membrane Transport Proteins; *Saccharomyces cerevisiae--metabolism--ME; *Saccharomyces cerevisiae Proteins; Amino Acid Sequence; Bacterial Proteins--genetics--GE; Base Sequence; Biological Transport, Active; DNA Mutational Analysis; Endoplasmic Reticulum--chemistry--CH; Endoplasmic Reticulum--metabolism --ME; Fluorescent Antibody Technique; Fungal Proteins--metabolism--ME; Heat-Shock Proteins--genetics--GE; Membrane Proteins--metabolism--ME; Molecular Sequence Data; Mutagenesis, Site-Directed; Protein Conformation; Recombinant Fusion Proteins; Saccharomyces cerevisiae--genetics--GE; Sequence Homology, Nucleic Acid; Subcellular Fractions--chemistry--CH

CAS Registry No.: 0 (Bacterial Proteins); 0 (DNAS protein); 0 (Fungal Proteins); 0 (Heat-Shock Proteins); 0 (Membrane Proteins); 0 (Membrane Transport Proteins); 0 (Recombinant Fusion Proteins); 0 (SEC63 protein, S cerevisiae); 0 (Saccharomyces cerevisiae Proteins)

Record Date Created: 19920806

Record Date Completed: 19920806

5/9/11 (Item 11 from file: 47)

02729516 SUPPLIER NUMBER: 03991138 (THIS IS THE FULL TEXT)

Multiple mechanisms of protein insertion into and across membranes.

Wickner, William T.; Lodish, Harvey F.

Science, v230, p400(8)
Oct 25, 1985
CODEN: SCIEAS ISSN: 0036-8075 LANGUAGE: ENGLISH
RECORD TYPE: FULLTEXT
WORD COUNT: 8165 LINE COUNT: 00661

TEXT:

Multiple Mechanisms of Protein Insertion Into and Across Membranes

Each of the 20 membrane-limited compartments of a mammalian cell contains a particular set of proteins that enables it to carry out its specific functions. The accurate and swift delivery of each protein to its correct compartment is an important step in gene expression. Except for the few proteins made within mitochondria and chloroplasts, protein synthesis begins with the formation of polysomes in the cytoplasm. An important concept that has guided recent work is that protein localization is initiated by binding to a specific receptor on an intracellular membrane. For different organelles, binding occurs either while the protein is still growing on the ribosome or after it is completed. Subsequent translocation of the protein into or across the membrane requires an input of energy. In

different cases this is provided either by a transmembrane electrochemical potential or by the folding of the protein during or after its translocation through the membrane. In many cases there is one or more "maturation' steps involving covalent modifications or folding on the opposite membrane surface.

Different integral membrane proteins --those bound to the phospholipid bilayer by hydrophobic interactions-- can have distinct asymmetric structures (Fig. 1). A protein may span the membrane once or several times, with the NH2- and COOH-terminus on either side of the bilayer. Our ideas of membrane assembly necessarily rest on our knowledge of the complexities of the structures of these proteins. Surprisingly different answers have emerged for various membrane proteins, organelles, and organisms, confounding early global hypotheses (1-3) and raising the need for better definition of fundamental mechanisms. In this article we focus on protein assembly into the endoplasmic reticulum, mitochondria, and the bacterial cell surface (Fig. 2). We do not discuss other assembly-competent organelles such as chlorooplasts, peroxisomes, and the nuclei, nor will we include organelles (such as Golgi or lysosomes) that are derived by membrane fission and fusion.

Secretory Proteins and the Endoplasmic Reticulum

Classic studies by Plalade and his colleagues (4) showed that secretory proteins are first found within the lumen of the rough endoplasmic reticulum (RER). Sealed fragments of the isolated organelle (rough microsomes) carry polysomes, which are highly enriched for secretory proteins and capable of chain completion in vitro (5-7). Nascent chains, emerging from the ribosome, cross the membrane (8, 9). In 1972, Milstein et al. (10) showed that a cell-free reaction, programmed by myeloma messenger RNA, synthesized a larger form of immunoglobulin light chains than was finally secreted from the cell. They proposed that this extra piece might be NH2-terminal and serve as a "signal' to direct secretion. Microsomes from these myeloma cells made mature-length protein, demonstrating that the signal sequence was cleaved during translocation into the RER. In 1975, Blobel and Dobberstein (1) showed that polysome-free dog pancreas microsomes would sequester newly made precursor of the immunoglobulin k light chain and proteolytically process it to its mature molecular weight if the microsomes were present during protein synthesis. "Signal sequences' were discovered at the NH2-termini of many nascent secretory proteins (11). They consist of 16-26 residues and have a polar, basic NH2-terminus and a central, apolar domain (12, 13).

Rothman and Lodish (14) used a synchronized cell-free synthesis of vesicular stomatitis virus (VSV) G protein to show that translocation requires membranes early in nascent chain growth. Walter and Blobel (15) and Meyer et al. (16) isolated two receptor proteins, the signal recognition particle (SRP) and the docking protein (DP), which coordinate the synthesis of nascent pre-secretory proteins (with an NH2-terminal signal peptide) with their insertion into microsomes (Fig. 3A). SRP is a complex of six polypeptides and 7S RNA (17). It binds to polysomes making pre-secretory or pre-membrane proteins and causes arrest of chain growth after approximately 80 residues. This is only a few more than the 40 residues buried within the ribosome plus the 25 (approximately) that comprise the average signal peptide. The SRP-polysome complex binds to DP, a 72,000-dalton integral membrane protein of the RER (16). Upon binding, the SRP is released and the polysome resumes chain elongation (Fig. 3A). The growing polypeptide chain passes through the endoplasmic reticulum membrane and into the lumen, where the signal peptide is cleaved and core glycosylation occurs.

In contrast to our present understanding of SRP-DP, less is known about the mechanism of polypeptide translocation through the apolar center of the endoplasmic reticular membrane. It is not known whether translocation is directly through lipid (2), as shown in Fig. 3, or through

a proteinaceous pore. Unlike translocation in mitochondria and bacteria, translocation into the RER does not require a transmembrane electrochemical potential. We propose that binding of the complex of SRP and nascent chain to the DP on the endoplasmic reticulum membrane would cause the membrane insertion of the signal sequence and the segment of amino acids adjacent to its COOH-terminus as a helical hairpin. SRP and DP would be dislodged as part of their recycling. Signal sequence cleavage of secretory proteins and many transmembrane glycoproteins occurs on the extracytoplasmic face of the membrane (Fig. 3A). This model incorporates features proposed previously by several investigators. Receptors to target nascent proteins as well as signal and "stop-transfer' or "membrane-anchor' sequences, and the idea that the information for the orientation of each membrane protein is contained in discrete, short sequences of the polypetide chain that act independently, are from the signal hypothesis. (1, 3, 18). The membrane trigger hypothesis (2) emphasized assembly information in the mature sequence, the importance of protein folding, and direct, hydrophobic interaction between the polypeptide chain and the hydrocarbon core of the membrane bilayer. The importance of the energetics of transfer of polypeptide domains between water and hydrocarbon was emphasized in the direct transfer model (19) and the helical hairpin hypothesis (20). The loop models (21, 22) and the helical hairpin hypothesis proposed that pairs of helices insert into the membrane.

Synthesis of Membrane Proteins on the Endoplasmic Reticulum

Biosynthesis of single-spanning membrane proteins whose NH2-termini face the extracytoplasmic face is similar to that of secretory proteins (Fig. 3A). These membrane proteins contain a sequence of about 20 hydrophobic amino acids that anchors the growing polypeptide in the phospholipid bilayer. For example, Yost et al. (23) and Guan and Rose (24) have shown that the apolar sequences at the COOH-terminus of immunoglobulin M (IgM) heavy chain and the VSV G protein stop translocation across the endoplasmic reticulum. These long stretches of hydrophobic amino acids could bind so tightly to the fatty acid core of the membrane that continued extrusion of the protein would be blocked; thus it would be considered a stop-transfer sequence. As the ribosomes continued elongation of the nascent chain, the newly added residues would remain on the cytoplasmic face. However, in some proteins long stretches of hydrophobic amino acids are translocated entirely across the bilayer (25); recognition of a stop-transfer sequence might also require an appropriate "receptor' protein in the endoplasmic reticulum.

Membrane proteins such as erythrocyte Band III (26), the histocompatibility antigen (HLA)-DR-associated invariant chain (27) and the asialoglycoprotein receptor (28), as well as a few secreted proteins such as ovalbumin (29) are synthesized on the endoplasmic reticulum, yet are not cleaved during insertion. This led to the concept of an internal, uncleaved signal sequence (3, 30), namely a sequence that could be recognized by the same proteins [except the leader (signal) peptidase] and would perform all the same functions as its cleaved counterparts. Studies with ovalbumin (26, 29) indicate that it has an uncleaved signal sequence near its NH2-terminus and have illustrated the difficulty in experimentally establishing the location of such an uncleaved signal sequence. One criterion is that such sequences be required for the insertion of a protein into microsomes (31, 32). Bos et al. (33) have provided the clearest demonstration of an uncleaved signal.

Uncleaved signal peptides, together with anchor sequences and other internal membrane insertion domains, can generate the complex topologies of different types of membrane proteins. For example, the bulk of the influenza virus neuraminidase is extracytoplasmic and is anchored to the membrane by a hydrophobic sequence at its NH2-terminus. Neuraminidase does not undergo endoproteolytic cleavage during its biosynthesis, and the NH2-terminal hydrophobic domain functions both as a signal and a membrane

anchor sequence. Genetic fusion of this NH2-terminal domain to a different viral glycoprotein that had its NH2-terminal (cleaved) signal deleted restored translocation of this glycoprotein (33). This suggests that neuraminidase has an NH2-terminal uncleaved signal sequence, and that all signal sequences, whether or not cleaved, function similarly in initiating **translocation** across the endoplasmic **reticulum** membrane. Similar studies (34) on the asialoglycoprotein receptor showed that the membrane spanning segment of 21 hydrophobic amino acids also functions as a signal sequence; SRP is required for its insertion into the RER. In Fig. 3B, we show how insertion of this protein might occur; as for secretory proteins, the signal peptide and the adjacent segment of the protein are postulated to insert as a helical hairpin (20).

Proteins such as cytochrome b5 are anchored to the membrane by apolar residues at the COOH-terminus, are synthesized on free polysomes, and probably insert into the endoplasmic **reticulum** membrane posttranslationally (Fig. 3C). SRP is not required for insertion of cytochrome b5 (35).

As proposed by Blobel (3), multi-spanning proteins such as sucrase-isomaltase and Band III could achieve their final topology by a succession of internal signal sequences and membrane-anchoring stop-transfer sequences. Hydrophobic side groups of the amino acids project outward to interact with the apolar fatty acyl core of the bilayer. However, many transmembrane sequences in multi-spanning proteins such as bacteriorhodopsin (36), Band III (37), or the acetylcholine receptor (38) have polar residues, which are not seen in the intramembrane segments of single-spanning proteins like VSV Gprotein, the asialoglycoprotein receptor, or M13 coat protein. These membrane-spanning helices may be amphipathic, with charged or polar residues confined to one face of the helix. Polar faces of several adjacent sequences could, in the mature protein, form a polar "pore' or "channel' through the membrane (36, 37, 39-41). These amphipathic helices may not be hydrophobic enough to function as simple signal or stop-transfer sequences. The proteins might utilize only one signal sequence, that would catalyze insertion of the most NH2-terminal helical hairpin of the nascent chain into the endoplasmic **reticulum** membrane, and one membrane anchor. As the nascent chain continued to grow in the cytoplasm, domains with hydrophobic surfaces could form and insert spontaneously into the membrane (2) without involvement of SRP and DP (Fig. 3C). Several helices could associate with each othe to shield their polar surfaces and form an "insertion domain' that could spontaneously insert into the phospholipid bilayer, presenting only an apolar face to the fatty acyl side chains of the phospholipids. Recombinant DNA techniques should allow the construction of novel membrane proteins that will test these concepts.

Demonstration of the close coupling between protein synthesis and membrane **translocation** in the endoplasmic **reticulum** has come from studies in a crude reticulocyte or wheat germ cellfree translation reaction supplemented with dog pancreas microsomes. Mutants, specific drugs, and in vivo studies, perhaps in a microorganism such as yeast, will be important to confirm or modify the current picture and to help in dissecting the crucial membrane **translocation** step.

Mitochondria

Mitochondria differ from the endoplasmic **reticulum** in almost every aspect of their biogenesis (41, 42). Except for the few proteins encoded by the mitochondrial DNA (43), all mitochondrial proteins are specified by nuclear genes. Each is synthesized in the cytoplasm and imported to one of the four mitochondrial compartments; outer membrane, intermembrane space, inner membrane, or matrix (Fig. 2). Isolated yeast and Neurospora mitochondria specifically imported only mitochondrial proteins from a cell-free translation reaction (44). Each of the proteins examined went to

its correct compartment, and the uptake was just as efficient when the mitochondria were added posttranslationally to the protein synthesis reaction as when they were present throughout the reaction (45). In vivo pulse-chase studies in Neurospora mycelia and yeast (46, 47) have shown that mitochondrial proteins pass through a cytoplasmic pool prior to binding to the organelle. Isolated mitochondria have polysomes on their outer surfaces, and these polysomes are highly enriched in nascent mitochondrial preproteins (48). However, Suissa and Schatz (49) showed that these represent only a small fraction of the polysomes for any given protein and that the proportion is governed simply by the rate of protein synthesis. Protein uptake into the matrix, inner membrane, or (except for cytochrome c) into the intermembrane space requires an electrochemical potential across the inner membrane (46, 50).

Many mitochondrial proteins are synthesized with a transient NH2-terminal leader peptide, while others are made and imported without cleavage (51). Mitochondrial leader peptides (52) are basic and have a different sequence pattern from pre-secretory proteins. Removal of mitochondrial leader peptides is catalyzed by a soluble matrix protease that has a specificity distinct from its counterpart in the endoplasmic **reticulum** or in Escherichia coli (53) (Fig. 4). In some cases, for proteins of the outer surface of the inner membrane or of the intermembrane space, the pre-sequence is removed by two successive proteolytic cleavages, one of which is catalyzed by the matrix protease (Fig. 4) (50, 54). Precursors of these proteins are thought to span the inner membrane transiently, with their NH2-termini facing the matrix space. This model would explain the puzzling requirement for an electrochemical potential across the inner membrane for **translocation** of several proteins ultimately located in the intermembrane space. As shown in Fig. 4, the second cleavage of these proteins is thought to occur at the outer surface of the inner membrane (52).

Mitochondrial pre-proteins use several outer membrane receptors for import (52, 57). Cytochrome c, a protein of the intermembrane space, is made as a precursor (apocytochrome) without the heme group and without a cleaved presequence. Uptake requires heme addition. Microgram amounts of apocytochrome (but not holocytochrome) block uptake of radioactive apocytochrome c, but not uptake of inner membrane or matrix proteins, suggesting the involvement of a specific receptor (58). A chimeric protein with 350 amino acids of the subunit of yeast F1 adenosine triphosphatase at its NH2-terminus and a large portion of -galactosidase at its COOH-terminus can be inserted into yeast mitochondria. This is also true for a gene fusion of the NH2-terminal 53 amino acids of the precursor to subunit IV of cytochrome oxidase and a different cytosolic enzyme, demonstrating that part of the NH2-terminus is sufficient to target a protein to the mitochondrion (59). Recently, several laboratories have reported that a soluble, cytosolic protein fraction is also necessary for import (60) (Fig. 4).

The outer membrane of mitochondria is distinct from the three other compartments in its biogenesis. Outer membrane proteins do not have cleaved leader sequences (61), and their membrane insertion does not require an electrochemical potential across the inner membrane. All of the information for targeting and anchoring the 70,000 molecular weight outer membrane protein is contained within the NH2-terminal 41 amino acids (62). Like the proteins of the internal mitochondrial compartments, assembly into the outer membrane is not coupled to translation.

Several fundamental questions of mitochondrial biogenesis are unanswered. What is the role of the electrical potential? Does import into the matrix involve separate steps of traversing the inner and outer membrane or, as illustrated in Fig. 4, does this occur at adhesion zones between the two membranes as previously suggested (41, 42)? What is the

number of outer membrane receptors, how do they catalyze translocation, and what other proteins are needed? Genetic identification of elements needed for import (63) may be vital to answering this question.

Bacterial Cell Surface

The cell surface of Gram-negative bacteria consists of three layers: the plasma membrane, an aqueous periplasm, and the outer membrane (Fig. 2). The mechanisms of membrane assembly and protein secretion in bacteria include those found in both mitochondria and RER. As in import of proteins into mitochondria, translocation of bacterial proteins across the plasma membrane requires a transmembrane electrochemical potential and, in different cases, can occur cotranslationally or after translation is complete. However, leader sequences of bacterial membrane and of exported proteins closely resemble those of the eukaryotic RER in structure (12, 64). Protein products of the bacterial sec (secretion) genes may, in some respects, have a function similar to that of the RER SRP. There is selective arrest of the synthesis of secretory proteins in secC mutants (65), even though translocation occurs late in translation or posttranslationally (as discussed below).

All known proteins of the periplasm and outer membrane, and at least several of the inner membrane, are made with NH2-terminal leader (signal) sequences (66). The majority of inner membrane proteins are made without a cleaved leader sequence (67, 68). Bacterial leader sequences are processed by a membrane-bound leader peptidase whose active site is on the periplasmic face of the plasma membrane (68) and whose substrate specificity is identical to that of the RER enzyme (69). However, unlike the RER, efficient bacterial protein export requires the membrane electrochemical potential (70). In this regard, bacterial export resembles the mitochondrial import process. Bacteria and mitochondria are also similar in the relative timing of protein synthesis and protein translocation across a membrane.

There has, until recently, been some confusion over whether bacterial proteins must begin crossing the plasma membrane early in their synthesis in order to be exported, as is seen in the RER, or whether their export is not coupled to polypeptide chain growth, similar to posttranslational import by mitochondria. Much of this confusion is semantic. "Cotranslational' refers to any event that occurs before the end of translation. However, it has been confused with an obligate coupled extrusion of the nascent chain through the bilayer as it emerges from the ribosome, as occurs in the eukaryotic RER. Such extrusion is apparently not seen in bacterial protein export, yet there are important cotranslational events, such as folding and interaction with sec-encoded proteins, which are necessary for later translocation.

Bacterial membrane fractions are enriched in polysomes coding for certain pre-secretory proteins (71). Davis and co-workers found that nascent chains of alkaline phosphatase could be labeled by a membrane-impermeant radioactive reagent added to intact cells or spheroplasts (72). M13 procoat, synthesized in a cell-free reaction (73), was shown to assemble efficiently into plasma membrane vesicles present during the synthesis of the protein (74). However, other experiments have shown that bacterial protein export normally does not occur early in the growth of the polypeptide chain. Ito et al. (75) observed that the pulse-labeling of the periplasmic or outer membrane proteins in intact cells was dramatically delayed relative to the labeling of cytoplasmic or inner membrane proteins. The export of -lactamase to the periplasm is entirely posttranslational (76), as is the insertion of M13 procoat protein into the plasma membrane in vivo (77). With the discovery that a membrane potential is needed for export, it was possible to experimentally separate the synthesis of both pro-OmpA and M13 procoat protein from their translocation (70).

These apparently contradictory observations, membrane-bound polysomes

and membrane-spanning nascent chains on the one hand and two-step export on the other, were resolved by the experiments of Randall (78). She demonstrated that nascent chains grow to at least 80 percent of their final size before they begin translocation through the plasma membrane. This critical chain length is different for each exported protein. Translocation does not then begin synchronously at the critical molecular weight, but, instead, a stochastic "race' ensues between polypeptide chain growth and protein translocation. Thus ribose-binding protein and pre- -lactamase are exported entirely posttranslationally, while only a fraction of the other protein species are exported cotranslationally. The remainder are completed as full-length pre-proteins within the cell, then are translocated entirely posttranslationally (79). These data are consistent with membrane-bound polysomes and with membrane-spanning nascent chains, yet clearly show that translocation is not strictly coupled to polypeptide chain elongation in bacteria.

Our current concept of protein export in bacteria is shown in Fig. 5. As with uptake of proteins into mitochondria, more than one receptor system targets different proteins to the bacterial plasma membrane. Mutations in any of several sec genes abolish export of a number of inner membrane, periplasmic, and outer membrane proteins but do not affect export of other proteins to these compartments (80). Since nascent pre-secretory proteins are not extruded through the bacterial membrane as they emerge from the ribosome, as in the RER, the sec proteins may serve functions distinct from those of SRP and DP. The sec proteins might stabilize certain pre-proteins until they can begin translocation. Alternatively, as illustrated in Fig. 5, they may bring specific proteins to the membrane early in their synthesis, analogously to SRP and DP. In bacteria, this may not lead to immediate translocation but may allow the polypeptide to grow at the interface between the aqueous cytoplasm and the apolar membrane.

Steps of Protein Translocation

Protein translocation in bacteria, mitochondria, and RER are summarized in Table 1. There are clearly no universal themes, or even completely consistent groupings of export themes. For example, bacterial pre-proteins have leader sequences like those of the endoplasmic reticulum but require a potential, as is seen for mitochondrial import. A new framework is needed for coherent organization of our knowledge of protein insertion into and across membranes. We suggest that the common features of protein translocation are its three necessary steps, the association of the protein with receptors on the correct membrane, the translocation through the membrane, and covalent modifications and folding on the opposite membrane surface. Individual proteins have evolved to use different combinations of the translocation themes to accomplish each step in export.

Protein binding to receptors on the correct membrane is essential to provide accurate protein sorting within the cell. In eukaryotic cells, recognition is mediated by specific soluble and organellebound elements. Bacteria, in which all exported proteins initially cross the same membrane, may only require sorting after translocation is complete. In addition to sorting, the binding step may stabilize pre-proteins against denaturation or possibly folding into a "dead-end' structure. This may be one function of the bacterial sec genes and of the mitochondrial cytoplasmic assembly component. This may even be viewed as a role of the SRP, which prevents elongation of the nascent chain unless DP is present. Receptors must facilitate translocation by either stabilizing the protein, catalyzing its refolding into a competent conformation, or transferring it to other elements that catalyze translocation.

Leader peptides of bacteria and RER (12, 13) average approximately 23 residues in length and have three characteristic domains. The NH2-terminal domain is short (1-5 residues), basic, and polar. The central domain is nonpolar and contains a "core' of 4-8 strongly hydrophobic residues. Mutations that after the charge at the NH2-terminus (81) or introduce

charged residues into the apolar domain (82) strongly inhibit protein export. The third domain begins with a helix-breaking residue (usually proline) and has small residues, characteristically glycine or alanine, at positions -3 and -1 relative to the cleavage site. These residues are not essential for translocation but serve as a leader peptidase recognition site (76, 83). Despite these conserved features, there is no true conservation of sequence.

The leader peptide is clearly essential for export (84, 85). It is not clear whether the leader is sufficient to specify export, that is, whether the match between leader sequence and the mature protein is critical, or whether part of the information for export lies in the sequence of the rest of the protein. Despite the similarities of the leader sequences, the answers to this question may be different for bacterial protein export and for protein secretion into the lumen of the RER. In bacteria, the fact that proteins grow to 80 percent of their final molecular weight before beginning translocation across the plasma membrane (78) suggests that there is information late in the protein sequence that is needed for secretion. This idea is supported by genetic studies; a fusion protein of the lamB leader joined to -galactosidase is not secreted across the plasma membrane (86), while a fusion product that contains virtually the entire lamB protein is efficiently secreted (87). A fusion of the -lactamase leader sequence to a foreign cytoplasmic protein (85) produced a chimeric protein that also failed to be secreted. Lipoprotein was still secreted when its leader sequence was replaced by that of OmpF (88); however, replacement of the rat pre-proinsulin leader sequence with the leader sequence from pre- -lactamase inhibited its secretion from E. coli (89). Mutations have been described in the mature portions of bacterial prolipoprotein (90), M13 procoat (91), and premaltose binding protein (92) that affect their export. On balance, it seems that the leader sequence is necessary, but not sufficient, for bacterial protein export.

In contrast, Yost et al. (23) have shown that the leader sequence of pre- -lactamase, fused to the membrane-spanning segment of IgM and the COOH-terminal portion of globin, will direct the insertion of the hybrid protein into dog pancreas microsomes. A chimeric protein consisting of the NH2-terminus of IgM heavy chain or of the asialoglycoprotein receptor (containing the internal signal) and the COOH-terminus of globin is completely translocated across the endoplasmic reticulum (23, 34). This suggests that insertion into the RER does not require information from the mature protein sequence. This difference between bacterial secretion and that in the RER is in accord with the differences in coupling between translation and translocation in these two systems.

The translocation step is not as well understood. Eukaryotic ribosomes appear to form a very tight junction with the RER membrane, and pre-secretory proteins may never contact the cytoplasm or fold prior to translocation. Thus, translocation of secretory proteins may be insensitive to the exact sequence of the polypeptide. In contrast, stoptransfer and insertion sequences are important information in the mature region of membrane proteins. Proper folding of soluble, secreted proteins that are synthesized on the RER may be essential for completion of translocation. Immediately after completion of synthesis, ribosomes release the nascent chain and dissociate into subunits. This leaves the COOH-terminal 25-35 amino acids (those formerly embedded in the large ribosomal subunit) exposed on the cytosolic face, and approximately 20 amino acids spanning the endoplasmic reticulum membrane. Since translocation of these last 45-55 residues cannot be coupled to chain elongation, some other process must provide a driving force. This could be the folding of the rest of the chain on the lumenal side of the membrane. This may be responsible for the SRP-independent translocation of short proteins such as the 70-residue precursor of bee venom mellitin (93) across microsomal membranes. The translation of this protein is virtually complete by the time the entire

signal sequence has emerged from the ribosome; its assembly into the microsome must therefore be essentially posttranslational.

What are the energetics of translocation and what is the role of the electrical potential? While answers to these questions must await further experiments, several facts are noteworthy. Bacterial pre-proteins are exported in a direction from the negative to positive with respect to the transmembrane electrochemical potential, while the opposite is true for the import of mitochondrial preproteins. If the mechanism underlying these requirements is the same, then it becomes difficult to envision a simple electrophoretic model. A mutant of M13 procoat has been described which, while unaltered in net charge in the translocated region, displays dramatically less dependence on the electrochemical potential for export (94). Bakker and Randall (95) showed that the chemical portion of the potential can substitute for the electrical component in driving bacterial export. This also casts doubt on simple electrophoretic models. Other possible roles for the potential include affecting the lipid structure, governing the concentration of other critical solutes, or even driving a protein-proton transport system. The well-studied voltage-dependent translocation of diphtheria toxin (96), mellitin (97), and asialoglycoprotein receptor (98) across lipid bilayers may be analogous to the translocation of preproteins; translocation of premitochondrial proteins and bacterial pre-secretory proteins need to be assayed in these systems.

When translated in a cell-free extract, the human erythrocyte glucose transporter can insert into the endoplasmic reticulum entirely posttranslationally (99). This glycoprotein probably spans the membrane as 12 -helixes (100) and bears a single N-linked oligosaccharide. The observation that its insertion into the endoplasmic reticulum membrane and its glycosylation requires SRP, but not concommitant translation, indicates that the binding and translocation steps need not be obligatorily coupled even in the RER. Its insertion thus resembles that of bacterial membrane proteins, except that there is no obvious requirement for a membrane potential.

The maturation step, which follows translocation, may be essential to the operation of additional sorting steps. Import of apocytochrome c across the mitochondrial outer membrane requires heme addition in the intermembrane space. When heme addition is blocked, the apocytochrome c remains bound on the outer mitochondrial surface (55). Explanations other than the reversibility of the insertion steps in the absence of maturation are, of course possible. For example, proteins catalyzing translocation might also need to donate pre-proteins to the appropriate maturation enzyme in order to catalyze another translocation event.

Prospectus

Further progress will depend on: (i) genetic and in vivo studies to define the physiological pathways and provide strains that are optimal for biochemical analysis, (ii) development of specific drugs to interrupt the pathway, reveal intermediates, and assist studies of enzymology, and (iii) analysis of cell-free reactions that are amenable to fractionation and reconstitution from their purified components. Bacterial export has benefited from intensive genetic study, while this approach is only beginning (in yeast) to be used in investigations of translocation into the RER and for mitochondrial biogenesis. Cloning has allowed the isolation of substantial quantities of the bacterial leader peptidase (101); it will allow preparation of large quantities of other catalysts of protein translocation in the near future. Mutants have been isolated to test the functions of different domains of pre-secretory and mitochondrial proteins. Pulse-labeling of a microorganism such as yeast may allow detection of predicted complexes, such as cytoplasmic SRP-polysomes, and may reveal new intermediates.

Cell-free translocation reactions (102) may provide assays for the

products of (sec) and protein localization (prl) genes (64) and allow the study of the role of the electrical potential. Submitochondrial translocation across isolated inner or outer membrane has not yet been reported, nor has a soluble detergent extract of RER been reconstituted to yield a translocation-competent liposome. Each of these cell-free reactions is the focus of intensive research and will provide further insights into the molecular mechanisms of translocation.

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Table: 1. Characteristics of the three steps of protein insertion into membranes.

Photo: Fig. 1. Topologies of integral membrane proteins. Segments of the chain within the bilayer are depicted as helices. Extramembrane regions are drawn as lines, and no attempt is made to depict the folding of these segments of the proteins. References for the amino acid sequences and transmembrane topologies are: VSV G protein (103); glycophorin (104); LDL (low-density lipoprotein) receptor (105); HLA-A heavy chain (106); influenza hemagglutinin (107); M13 coat protein (108); influenza neuraminidase (109); asialoglycoprotein receptor (28, 111); transferrin receptor (112); bacterial leader peptidase (68); HLA-DR invariant chain (27); acetylcholine receptor subunit (41); cytochrome b5 (113); erythrocyte Band III (37, 114). Although only six membrane-spanning regions are drawn, recent data on the sequence of the entire Band III messenger RNA (37) indicates that there are 12 stretches of hydrophobic residues of length sufficient to span the membrane. Initial work suggested that the precursor of sucrase-isomaltase spanned the plasma membrane twice, with both the NH2- and COOH-termini remaining exoplasmic (110). However, the complete complementary DNA sequence, as well as other data, indicate only a single membrane-spanning segment, with the NH2-terminus facing the cytoplasm (115). N, NH2-terminus; C, COOH-terminus.

Photo: Fig. 2. Compartments of endoplasmic reticulum, mitochondria, and bacteria.

Photo: Fig. 3. A model for cotranslational insertion of membrane and secreted proteins into or through the endoplasmic reticulum membrane. (A) Secreted proteins and VSV G protein. Binding of the complex of SRP and nascent chain to the DP on the endoplasmic membrane would cause the insertion into the endoplasmic reticulum membrane of the signa sequence (hatched box) and the segment of amino acids adjacent to its COOH-terminus as a helical hairpin. After the nascent chain is cleaved, continued extrusion across the endoplasmic reticulum membrane would generate a secretory protein or, if there is an anchor (or stop-transfer) sequence, a

single-spanning transmembrane protein with the same conformation as the VSV G protein. (B) Asialoglycoprotein receptor. If the signal sequence is uncleaved and there is no anchor sequence, continued growth and translocation of the nascent chain across the endoplasmic reticulum membrane would generate a protein with its NH2-terminus facing the cytoplasm and its COOH-terminus in the lumen of the RER. (C) The synthesis of proteins such as cytochrome b5 occurs on cytoplasmic polysomes. The completed protein then inserts spontaneously into the RER, without mediation of SRP or DP, by means of an insertion sequence or domain. (D) Multi-spanning membrane proteins. The first helical hairpin could result from a combination of signal and anchor sequences; subsequent helices could fold against each other, forming a domain that would insert spontaneously as the peptide grows in the cytoplasm. The cylinders represent a possibly alternative structure of a membrane-spanning -helix.

Photo: Fig. 4 (left). Protein import into mitochondria. The import of a matrix protein and an intermembrane protein with cleaved leader sequences is depicted. The function of the cytoplasmic import element, a 40-kilodalton protein (60), is not known. Proteins of the intermembrane space may partially insert across the inner membrane and undergo two-step proteolysis. R represents the outer membrane receptors for pre-mitochondrial proteins. P1 and P2 are proteases that remove the mitochondrial leader peptide. Fig. 5 (right). Assembly of the bacterial cell surface. The leader peptide is indicated by a hatched rectangle. As discussed in the text, many (but not all) pre-proteins require functional sec genes for export. Proteins reach critical molecular weight (78) or full length prior to beginning potential-dependent translocation.
CAPTIONS: Topologies of integral membrane proteins. (chart); Compartments of endoplasmic reticulum, mitochondria, and bacteria. (chart); Model for cotranslational insertion of membrane and secreted proteins into or through the endoplasmic reticulum membrane. (chart); Protein import into mitochondria. (chart); Characteristics of the three steps of protein insertion into membranes. (table)

SPECIAL FEATURES: illustration; chart; table
DESCRIPTORS: Protein biosynthesis--Research; Membrane proteins--Genetic aspects; Mitochondrial membranes--Genetic aspects; Proteins--Genetic aspects
FILE SEGMENT: MI File 47

5/9/10 (Item 10 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

10884565 PMID: 7876253
Intracellular folding of tissue-type plasminogen activator. Effects of disulfide bond formation on N-linked glycosylation and secretion.
Allen S; Naim H Y; Bulleid N J
University of Manchester, School of Biological Sciences, United Kingdom.
Journal of biological chemistry (UNITED STATES) Mar 3 1995, 270 (9) p4797-804, ISSN 0021-9258 Journal Code: 2985121R
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS

The addition of N-linked core oligosaccharides to membrane and secretory glycoproteins occurs co-translationally at asparagine residues in the tripeptide sequon Asn-Xaa-Ser/Thr soon after **translocation** of the nascent polypeptide into the lumen of the endoplasmic **reticulum** . However, the presence of the sequon does not automatically ensure core glycosylation, as many proteins contain sequons that remain either unglycosylated or glycosylated to a variable extent. To investigate whether intracellular protein folding can influence sequon utilization, we have expressed tissue-type plasminogen activator (t-PA) in **cell** culture in the presence of mild concentrations of the reducing agent dithiothreitol to prevent co-translational **disulfide** bond formation in the endoplasmic **reticulum** . We show that conditions that prevent **disulfide** bond formation lead to complete glycosylation of a sequon that otherwise undergoes variable glycosylation in untreated cells. This demonstrated that folding and **disulfide** bond formation of t-PA determines its extent of core N-linked glycosylation. When dithiothreitol was removed from the cells, the reduced and overglycosylated t-PA formed **disulfide** bonds, folded, and was secreted. We also show t-PA present within cells is more susceptible to reduction with low concentrations of dithiothreitol than secreted t-PA.

Tags: Research Support, Non-U.S. Gov't

Descriptors: *Disulfides--chemistry--CH; *Protein Folding; *Tissue Plasminogen Activator--chemistry--CH; Amino Acid Sequence; Animals; Biological Transport; CHO Cells; Dithiothreitol--pharmacology--PD; Endoplasmic Reticulum--drug effects--DE; Endoplasmic Reticulum--metabolism --ME; Glycosylation; Hamsters; Molecular Sequence Data; Tissue Plasminogen Activator--metabolism--ME; Tissue Plasminogen Activator--secretion--SE

CAS Registry No.: 0 (Disulfides); 3483-12-3 (Dithiothreitol)

Enzyme No.: EC 3.4.21.68 (Tissue Plasminogen Activator)

Record Date Created: 19950405

Record Date Completed: 19950405

5/9/4 (Item 4 from file: 47)

03634617 SUPPLIER NUMBER: 11733790 (THIS IS THE FULL TEXT)

Recombinant toxins for cancer treatment.

Pastan, Ira; FitzGerald, David

Science, v254, n5035, p1173(5)
Nov 22, 1991
CODEN: SCIEAS ISSN: 0036-8075 LANGUAGE: ENGLISH
RECORD TYPE: FULLTEXT; ABSTRACT
WORD COUNT: 4745 LINE COUNT: 00370

ABSTRACT: Conventional chemotherapy has made great strides in treating cancer over the last three decades. Some forms of cancer, such as certain leukemias, may be cured by chemotherapy; patients with other forms of cancer, such as ovarian cancer, may enjoy longer survival as a result of adjuvant chemotherapy. However, for many forms of cancer, satisfactory treatment does not exist. Among the potential treatments that are under investigation are recombinant toxins. The rationale for this form of treatment is to target the toxic therapy directly to the cancer cell. For example, if a cancer cell expresses a large number of epidermal growth factor receptors on its cell surface, a toxic molecule might be attached to a molecule of epidermal growth factor itself. The growth factor increases the likelihood that the molecular complex will stick to a cancer cell; the toxin then kills the malignant cell. Similar strategies may be applied to specific cell surface antigens on cancer cells. Antibodies that recognize

these cancer cell antigens may be used to provide the specific attachment to cancer cells. Similarly, toxins attached to these antibodies kill the cancer cell. The most practical method for making these toxic hybrids is genetic engineering. The molecules that specifically bind to cells are protein molecules; the techniques of molecular biology may be used to construct hybrid molecules in which a protein toxin is attached. Protein toxins include diphtheria toxin, produced by the diphtheria bacteria; ricin, produced by the castor bean; and Pseudomonas endotoxin. These toxins naturally occurring toxins are among the most poisonous substances known. (Consumer Summary produced by Reliance Medical Information, Inc.)

AUTHOR ABSTRACT: Recombinant toxins target cell surface receptors and antigens on tumor cells. They kill by mechanisms different from conventional chemotherapy, so that cross resistance to conventional chemotherapeutic agents should not be a problem. Furthermore, they are not mutagens and should not induce secondary malignancies or accelerate progression of benign malignancies. They can be mass-produced cheaply in bacteria as homogenous proteins. Either growth factor-toxin fusions or antibody-toxin fusions can be chosen, depending on the cellular target.

TEXT:

RECOMBINANT TOXINS ARE HYBRID CYTOTOXIC PROTEINS made by recombinant DNA technology that are designed to selectively kill cancer cells. The cell-targeting moiety can be a growth factor or a single chain, antigen-binding protein. The toxic moiety is a portion of a bacterial or plant toxin. Immunotoxins are similar in concept but are composed of antibodies chemically linked to toxins.

More than 30 years ago chemotherapeutic drugs began to be used to treat cancer as a supplement to surgery and radiation therapy. Now that several decades have passed, it is clear that the current generation of chemotherapeutic drugs can achieve cures of certain leukemias and lymphomas and, in the adjuvant setting, prolong the lives of patients with breast cancer, ovarian cancer, and several other types of cancer. Because chemotherapy is not a cure for the common types of cancer in adults, new therapies must be developed.

One approach is to target a cytotoxic agent to the cancer cell [1]. To accomplish this, the cytotoxic agent is attached to an antibody or a growth factor that preferentially binds to cancer cells. The targets for this type of therapy can be growth factor receptors, differentiation antigens, or other less characterized cell surface antigents. It is now established that many cancers overproduce growth factor receptors that can function as oncogenes and promote the growth of the cancer cells [2-4]. For example, the epidermal growth factor receptor is present in large amounts (up to 3 x [10.sup.6] receptors per cell) in many squamous cell and epidermoid carcinomas, glioblastomas, and some metastatic ovarian and bladder cancers [5-7]. Normal cells contain as many as 3 x [10.sup.5] receptors per cell [8]. The interleukin-2 (IL-2) receptor is present in substantial numbers on the cells of patients with adult T cell leukemia (ATL; 3 x [10.sup.4] receptors per cell) and in lower numbers in various other lymphoid malignancies [9].

Differentiation antigens that occur on normal cells such as B lymphocytes are often also present on tumor cells such as B cell lymphomas. Because such antigens are not present on the stem cells that produce B cells, any mature B cells that are killed by targeted therapy will be replaced from the stem cell population, whereas the cancer cells will not be replaced [10]. Finally, there are antigens preferentially expressed on cancer cells whose functions are not yet understood. some of these, such as carcinoembryonic antigens [11], are fetal antigens, which are either not present or only present in small amounts on normal adult tissues. This group also contains antigens of unknown origin that are only defined by their reactivity with a monoclonal antibody [12-14].

For targeted drug delivery to be successful, it is necessary that the cytotoxic agent be extremely active. Bacterial and plant toxins, which are some of the most cytotoxic substances known, act by irreversibly arresting the synthesis of protein in eukaryotic cells. Pseudomonas exotoxin (PE) and diphtheria toxin (DT) do this by enzymatically inactivating elongation factor 2, an essential component of protein synthesis. Ricin and other plant toxins cleave a glycosidic bond in 28S ribosomal RNA (rRNA), thereby destroying the ability of ribosomes to synthesize protein. Because these toxins are catalysts with high turnover numbers, few molecules need to reach the cytoplasm to kill the target cell. These types of toxins enter cells by endocytosis and are processed to an active fragment that translocates across the cell membrane into the cytosol where the components of protein synthesis are located (Fig. 1). If one bypasses this pathway and directly injects an activated form of DT or PE into the cytosol, only the injection of a few molecules is necessary to kill the cell [15].

Initially, agents that delivered toxins to cancer cells were constructed by chemically coupling antibodies to toxins [16-19]. Immunotoxins have been made by the coupling of antibodies to ricin A chain or to several other plant toxins and to modified forms of DT and PE. More recently, genetic engineering has been used to make recombinant toxins by fusing modified toxin genes to DNA elements encoding growth factors or the combining regions of antibodies [20-23]. These chimeric genes are then expressed in Escherichia, coli, from which the recombinant toxins are prepared.

Toxins in their native form require a minimum of three biochemical functions to kill cells: cell binding, cytotoxicity, and the ability to translocate the toxic activity into the cytosol. An advance in understanding the structural basis of each of these functional regions occurred with the crystallization of PE and the elucidation of its three-dimensional structure [24]. PE is a single polypeptide chain with a size of 66 kD that is arranged into three major structural domains. This arrangement suggested that each domain could be responsible for one function. To test this hypothesis, Hwang et al. [25] isolated the PE gene from Pseudomonas aeruginosa and used a bacterial expression system to produce the whole toxin or fragments of the toxin corresponding to each structural domain [25]. The results showed that domain Ia (amino acids 1 to 252) was the cell binding domain, domain II (amino acids 253 to 364) was required for translocation, and domain III (amino acids 400 to 613) was required for the adenosine diphosphate (ADP) ribosylation and inactivation of elongation factor 2. A subdomain termed Ib, which is composed of amino acids 365 to 399, has no known function, and most of it can be deleted without loss of activity [26].

The steps involved in the killing of cells by PE are shown in Fig. 1. DT probably follows a similar pathway. Soon after toxin binding, the toxin-receptor complex is internalized by the pathway of receptor-mediated endocytosis [27, 28]. The toxin travels via clathrin-coated pits into endocytic vesicles where the toxin is cleaved into two pieces by a combination of a proteolytic step and reduction of a disulfide bond [29]. The 37-kD fragment derived from the COOH portion, which contains part of domain II and all of domain III is translocated to the cytosol where it arrests protein synthesis and causes cell death [29]. Although Fig. 1 illustrates translocation occurring from an endocytic vesicle, recent data suggest translocation may occur in the endoplasmic reticulum.

Diphtheria toxin is also a single chain toxin in which the functional domains are arranged in the opposite order from PE, with the ADP ribosylating function at the [NH.sub.2]-terminus and the binding domain at the COOH-terminus [30, 31]. DT made in E. coli is thought to be proteolytically cleaved after binding to target cells [32]. Ricin is composed of two subunits linked together by a disulfide bond. The A chain contains the enzymatic activity; the B chain binds to galactose residues

present on many different cell surface glycoproteins and glycolipids. Pseudomonas exotoxin, diphtheria toxin, and ricin are each synthesized as a single polypeptide chain. The chain is later proteolytically cleaved into two fragments, and the fragment containing the toxic enzymatic activity is translocated

Immunotoxin	Disease	Phase	References
Xomazyme-Mel-RTA	Melanoma	1/2	(83)
791T/36-RTA	Colorectal cancer	1	(56)
260 F9-RTA	Breast cancer	1	(54, 55)
Anti-B4-B-ricin	B **cell** malignancies	1/2	(37 (*), 84 (*))
T-101-RTA	CLL/T-ALL	1	(18, 85)
Anti-CD22 RTA dgRTA	B **cell** malignancies	1	(53)
Anti-CD22 Fab dgRTA	B **cell** malignancies	1	(53)
Anti-Tac(Fv)-PE40	ATL	1	(86 (*))
OVB3-PE	Ovarian cancer	1	(57)
DAB 486-IL-2	ATL and other lymphoid tumors	1	(21 (*))

(*) Results of clinical trials are unpublished.

into the cytosol [29, 32]. PE is not cleaved (processed) until it enters the target **cell** where both proteolytic cleavage and **disulfide** bond reduction occur. In contrast, diphtheria toxin is clipped shortly after secretion, and ricin is cleaved within the seed where it is synthesized [33]. In both examples the two fragments remain held together by a **disulfide** bond which is ultimately broken within the target **cell** [34].

Immunotoxins

To construct active immunotoxins, the toxin must be modified so that its interactions with cellular receptors are diminished or abolished (Fig. 2). As a consequence, toxin entry is mediated by antibody binding. with ricin, this is accomplished through removal of the B chain [35], blockage of the galactose binding site [36] or attachment of a galactose-rich carbohydrate to the B chain [37]. With PE, this modification occurs when the antibody is coupled to domain I of PE, which interferes with the binding of domain I to the PE receptor [38], or when domain I (amino acids 1 to 252) is genetically deleted and coupled to antibody to domain II [39]. With DT, this modification occurs through the mutation of a key amino acid in the binding domain near the COOH-terminus or through the removal of a portion of the COOH-terminus that is responsible for binding to cells [40, 41].

Ricin is purified from castor beans and has been available for immunotoxin production for many years. More recently, a recombinant form of the A chain has been produced in E. coli [42]. PE has been obtained from the culture medium of P. aeruginosa, but both the whole toxin and mutant forms have been produced in E. coli [20] as have been mutant forms of DT.

The activity of an immunotoxin is initially assessed by measuring its ability to kill cells with target antigens on their surfaces. Because toxins act within the **cell** , receptors and other surface proteins that naturally enter cells by endocytosis usually make good targets for immunotoxins, but surface proteins that are fixed on the **cell** surface do not. However, if several antibodies recognizing different epitopes on the same **cell** surface protein are available, it is useful to test them all, because some, perhaps by producing a conformational change in the target protein's structure, may induce its internalization or direct its intracellular routing to an appropriate location for toxin translocation [43, 44]. Also, it is possible to induce internalization of a target surface protein if the immunotozin contains a form of PE or ricin in which the binding of the toxin moiety to its receptor, although weakened by chemical modification, still occurs and promotes internalization since toxin receptors are efficiently internalized [14, 37, 41].

Many immunotoxins produce selective killing in cell culture, but only a few of these have been able to cause substantial or complete tumor regression in animals. Ricin A chain coupled to antibodies recognizing B cell specific antigens have caused complete regression of B cell lymphomas in mice [45]. When antibodies to carcinomas are used, only partial responses have been observed [46-48]. Regression of human carcinomas growing in immunodeficient mice has been achieved by treatment with monoclonal antibodies reacting with ovarian, colon, and breast cancers coupled either to PE itself or to PE40, a mutant form of PE in which the cell binding domain was deleted [14, 49, 50]. One of these, B3-PE40, causes complete regression of human tumors growing in mice [51].

Several immunotoxins have been developed and approved for human trials. Two different kinds of trials have been conducted. The first, involves the ex vivo addition of immunotoxins to harvested bone marrow to eliminate contaminating tumor cells before reinfusion in patients undergoing autologous bone marrow transplantation. A variety of antibodies, linked to ricin or ricin A chain, including anti-CD5 and anti-CD7, have been used for this purpose [52]. The second kind of trial involves the parenteral administration of immunotoxins, either regionally (such as the peritoneal cavity) or systemically to patients with cancer. These have been primarily Phase 1 and 2 trials in patients in which conventional treatments have failed, and the patients have a large tumor burden. A list of clinical trials, either completed or still in progress, is provided in Table 1. Definite responses have been noted with lymphomas, and these trials will be expanded [53]. So far, the antibodies used for the preparation of immunotoxins to treat carcinomas or other solid tumors have been found to react with important normal human tissues (such as neural tissue and bone marrow) and produce dose-limiting toxicity without significant clinical responses [54-57].

Genetically Engineered Recombinant Toxins

The production of immunotoxins by chemical coupling methods is expensive because it requires large amounts of antibody and toxin. Furthermore, the chemical conjugation methods used produce heterogeneous products, and antigen binding is often affected by chemical derivatization. It has been possible to overcome these difficulties and to create cytotoxic agents by genetic engineering. Both PE and DT have been used to make recombinant toxins in E. coli [20, 23]. Ricin-based molecules have been difficult to produce probably because the A chain of the plant toxin must be attached to the cell recognition domain by a disulfide bond, and disulfide-linked subunits are difficult to produce in bacteria. The addition of a proteolytic cleavage sequence may help to overcome this difficulty [58].

The x-ray crystallographic structure of PE has been used as a guide for the synthesis of genetically engineered recombinant toxins (Fig. 3). The specific binding of PE to target cells occurs through an interaction of domain I with cellular PE receptors [59, 60]. The junction of domain I with domain II occurs between [Glu.sup.252] and [Gly.sup.253]. Therefore, in constructing recombinant toxins, domain I was deleted, and the COOH-terminal-amino acid of various growth factors and other targeting molecules were fused directly to [Gly.sup.253] of PE (occasionally a few additional amino acids have been added as a link between the COOH-terminus of the growth factor and [Gly.sup.253] of PE to make cloning more convenient). One widely studied molecule is TGF-[alpa]-PE40, which was constructed by replacing domain I of PE with transforming growth factor alpha (TGF-[alpha]). In this chimeric toxin, the 23-kD domain I is replaced by the 6-kD growth factor (Fig. 3), to produce a chimeric toxin that selectively binds to and kills cells with epidermal growth factor (EGF) receptors. The structure of the plasmid encoding TGF-[alpha]-PE40 is shown in Fig. 4 with the cDNA encoding TGF-[alpha] inserted adjacent to the PE40 gene. The expression vector used for the production of TGF-[alpha]-PE40 and

other PE-based chimeric toxins in E. coli contains the bacteriophage T7 promoter, an efficient ribosome binding site and an Nde I site (CATATG), which encodes a methionine initiation codon where targeting ligands can be conveniently inserted. The gene encoding the phage T7 polymerase is inserted into the E. coli chromosome next to a lac promoter so that it can be induced by the addition of isopropylthiogalactoside (IPTG) [61].

TGF-[alpha]-PE40, like other chemieric toxins made in E. coli, accumulates in large amounts within the cell in insoluble aggregates (inclusion bodies). After cell disruption, inclusion bodies are easily isolated and can contain up to 90% recombinant protein in an insoluble form. The protein is then dissolved in a strong denaturant such as 7 M guianidine-HCl, renatured, and can be

[TABULAR DATA OMITTED]

purified to near homogeneity in two or three steps by conventional column chromatographic methods [62]. TGF-[alpha]-PE40 binds to EGF receptor-containing cells with about the same affinity as TGF-[alpha], and its toxicity on these cells is directly related to the number of receptors present.

Recombinant Toxins and Their Targets

The EGF receptor (EGF-R) has been the subject of intense study and shown to act as an oncoprotein when it is overexpressed in normal cells [2]. TGF-[alpha]-PE40 is extremely cytotoxic to cells that contain EGF receptors and has been shown to have an antitumor effect in animals bearing tumors that have more EGF receptors than normal cells [50, 63]. Clinical trials have just begun in which TGF-[alpha]-PE40 is instilled into the urinary bladder to treat superficial bladder cancer. Recombinant toxins have now been made by combining IL-2, IL-4, IL-6, IGF-1, and acidic fibroblast growth factors (FGF) with PE40 [62, 64-67] and DT with IL-2 and melanocyte-stimulating hormone [21, 68] (Fig. 3). Each was cytotoxic to cell lines containing the appropriate receptors. Furthermore, anti-tumor activity in animals bearing human cancers has been demonstrated with TGF-[alpha]-PE40, IL-2-PE40, and IL-6-PE40 [69, 70]. Another way to assess efficacy of such agents is to test them against cancer cells directly isolated from patients. DT-IL-2 has activity against fresh leukemic cells from patients with ATL [71] and is now being evaluated in Phase 1 trials in individuals with various lymphoid malignancies. Table 2 summarizes some tumor types that contain large numbers of receptors and may serve as targets for recombinant toxin therapy.

Although growth factors fused to toxins have proved to be effective cytotoxic agents, a concern has been raised that some of these might promote tumor growth in certain circumstances, for example, if less than a full toxic dose were administered [65, 72, 73]. Because antibodies to growth factor receptors do not usually have agonist effects, the antigen combining region of these antibodies can be used for targeting. This approach takes advantage of the finding that the variable regions of the light and heavy chains of antibodies can be combined into a single chain form that retains high affinity binding to antigen [74-76]. Accordingly, a complementary DNA (cDNA) from the antibody to Tac (anti-Tac) was used to construct an Fv fragment that was fused to PE40 [22] (Fig. 3). The Fv portion of an antibody molecule is composed of two variable regions of the light and heavy chains and these can be held together by a linking peptide to make a single chain Fv. This single-chain recombinant toxin anti-Tac-(Fv)-PE40, which is under preclinical development has potent cell-killing activity against cells with IL-2 receptors and against cells directly isolated from ATL patients [77]. The approach, of combining single chain antibodies with PE40, has been extended to several other antibodies, including an antibody to the human transferrin receptor [78], OBV3 [79], and B3 [80]. The antibody to B3, which binds to a carbohydrate antigen expressed on the surface of many carcinomas [81], has been used to make a single-chain recombinant toxin that causes the complete regression of human

tumors in mice [80].

Problems and Prospects

Toxins are foreign proteins and highly immunogenic. Therefore, in the absence of immunosuppression, neutralizing antibodies develop about 10 days after exposure to toxin [57, 82] and antibodies to DT already exist in most individuals who have received immunizations with diphtheria, pertussis, and tetanus (DPT). Animal studies have shown that immunotoxins and recombinant toxins act quickly so that tumors regress in a few days. Similar rapid responses should occur in humans. Nevertheless for long-term therapy, concomitant administration of immunosuppressive agents will be necessary to prolong the treatment period or the same antibody or ligand can be used to target different toxins. Many of the patients being treated in the current clinical trials have received extensive chemotherapy and are severely immunosuppressed so that the treatment period can be extended. In this review, we have discussed the utility of three toxins that are under clinical development, but there are still concerns associated with their use. Many people in the Western world have neutralizing antibodies to DT as a result of childhood immunization, and this has caused concern about the utility of recombinant toxins containing DT. Ricin A chain and blocked ricin both make active immunotoxins, but the recombinant molecules produced so far have low cytotoxic activity. The advantage of PE is that its structure is known. And, by means of this as a guide, the cell binding domain has been successfully replaced with more than a dozen different ligands including several different single chain antibodies. Furthermore, less than 3 percent of humans have pre-existing antibodies to PE. In the next several years, we anticipate that the major role of immunotoxins and recombinant toxins will be in the adjuvant setting for the treatment of metastatic disease that remains after surgery or radiation therapy.

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The authors are in the Laboratory of Molecular Biology, Division of Cancer Biology, Diagnosis, and Centers, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.
CAPTIONS: Journey to the cytosol. (chart); Strategies for making immunotoxins. (chart); Clinical trials with immunotoxins. (table); Strategies for making recombinant toxins. (chart); Expression of recombinant toxins from plasmids containing the T7 promoter. (chart); Targets for recombinant toxins. (table)

SPECIAL FEATURES: illustration; chart; table
DESCRIPTORS: Chemotherapy--Research; Recombinant molecules--Therapeutic use; Cancer--Chemotherapy; Toxins--Therapeutic use; Antibody-toxin conjugates--Therapeutic use
FILE SEGMENT: MI File 47
? t s5/3/1-3

5/3/1 (Item 1 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00322968

A process for the production of transgenic plants with increased nutritional value via the expression of modified 2S storage albumins in said plants

Verfahren zur Herstellung transgenetischer Pflanzen mit erhohtem Nahrwert durch Expression von modifizierten 2S-Speicheralbuminen in diesen Pflanzen

Procede pour la production de plantes transgeniques de valeur nutritionnelle augmentee par expression des albumines de stockage 2S modifiees dans ces plantes

PATENT ASSIGNEE:
PLANT GENETIC SYSTEMS, N.V., (654072), Kolonel Bourgstraat 106 Bus 1, B-1040 Brussel, (BE), (applicant designated states: AT;BE;CH;DE;ES;FR;GB;GR;IT;LI;LU;NL;SE)
EMPRESA BRASILEIRA DE PESQUISO AGROPECUARIA - EMBRAPA, (1199590), S.A.I.N. Parque Rural, BR-70770 Brasilia DF, (BR), (applicant designated states: AT;BE;CH;DE;ES;FR;GB;GR;IT;LI;LU;NL;SE)
INVENTOR:
De Clercq, Ann, Berkenlaan 130, B-8730 Harelbeke, (BE)
Krebbers, Enno, 1724 Glenview, Alhambra California 91803, (US)
Vandekerckhove, Joel Stefaan, Rode Beukendreef 27, B-8021 Loppem, (BE)
barreto De Castro,Luiz, Shin Qi 14 Conjunto 05 Casa 17, Brasilia DF,71500 , (BR)
Gander,Eugen, SQS 409 Bloco B Entrada B Apt. 202, Brasilia DF,70258, (BR)
Van Montagu,Marc, De Strassartstraat 120, B-1050 Brussels, (BE)
LEGAL REPRESENTATIVE:
Gutmann, Ernest et al (15992), Ernest Gutmann - Yves Plasseraud S.A. 3, rue Chauveau-Lagarde, F-75008 Paris, (FR)
PATENT (CC, No, Kind, Date): EP 318341 A1 890531 (Basic)
EP 318341 B1 960731
APPLICATION (CC, No, Date): EP 88402650 881020;
PRIORITY (CC, No, Date): EP 87402348 871020; EP 88402611 881014
DESIGNATED STATES: AT; BE; CH; DE; ES; FR; GB; GR; IT; LI; LU; NL; SE
INTERNATIONAL PATENT CLASS: C12N-015/00; C12N-005/00; A01H-001/00
ABSTRACT WORD COUNT: 155

LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS A	(English)	EPABF1	1094
CLAIMS B	(English)	EPAB96	535
CLAIMS B	(German)	EPAB96	480
CLAIMS B	(French)	EPAB96	566
SPEC A	(English)	EPABF1	9111
SPEC B	(English)	EPAB96	9344
Total word count - document A			10206
Total word count - document B			10925
Total word count - documents A + B			21131

5/3/2 (Item 2 from file: 348)
DIALOG(R)File 348:EUROPEAN PATENTS

00322964

A process for the production of biologically active peptide via the expression of modified storage seed protein genes in transgenic plants
Verfahren zur Herstellung eines biologisch-aktiven Peptides durch Expression von modifizierten Speicherproteingenen in transgenetischen Pflanzen
Procede pour la production de peptide biologiquement actif par l'expression de genes de proteines de reserves modifiees dans les plantes transgeniques

PATENT ASSIGNEE:
PLANT GENETIC SYSTEMS N.V., (654070), Kunstlaan Avenue des Arts, 46, B-1040 Bruxelles, (BE), (applicant designated states: AT;BE;CH;DE;ES;FR;GB;GR;IT;LI;LU;NL;SE)
INVENTOR:
Vandekerckhove, Joel Stefaan, Rode Beukendreef 27, B-8021 Loppem, (BE)
Krebbers, Enno, 1724 Glenview, Alhambra, CA 91803, (US)
Botterman, Johan, Het Wijngaardeke 5, B-9721 Zevergem-De Pinte, (BE)
Leemans, Jan, P. de Denterghemlaan 2, B-9831 Deurle, (BE)
LEGAL REPRESENTATIVE:
Gutmann, Ernest et al (15992), Ernest Gutmann - Yves Plasseraud S.A. 3, rue Chauveau-Lagarde, 75008 Paris, (FR)
PATENT (CC, No, Kind, Date): EP 319353 A1 890607 (Basic)
EP 319353 B1 961002
APPLICATION (CC, No, Date): EP 88402646 881020;
PRIORITY (CC, No, Date): EP 87402348 871020
DESIGNATED STATES: AT; BE; CH; DE; ES; FR; GB; GR; IT; LI; LU; NL; SE
INTERNATIONAL PATENT CLASS: C12N-015/62; C12N-015/82; C07K-014/415; C07K-014/60; C07K-014/70;
ABSTRACT WORD COUNT: 240
LANGUAGE (Publication,Procedural,Application): English; English; English
FULLTEXT AVAILABILITY:

Available Text	Language	Update	Word Count
CLAIMS A	(English)	EPABF1	1225
CLAIMS B	(English)	EPAB96	831
CLAIMS B	(German)	EPAB96	722
CLAIMS B	(French)	EPAB96	906
SPEC A	(English)	EPABF1	19571
SPEC B	(English)	EPAB96	18420
Total word count - document A			20797
Total word count - document B			20879
Total word count - documents A + B			41676

5/3/3 (Item 3 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00185463 **Image available**
RECOMBINANT RETROVIRUSES DELIVERING VECTOR CONSTRUCTS TO TARGET CELLS
RETROVIRUS DE RECOMBINAISON APPORTANT DES CONSTRUCTIONS DE VECTEUR A DES CELLULES CIBLES
Patent Applicant/Assignee:
VIAGENE INC,
Inventor(s):
GRUBER Harry E,
JOLLY Douglas J,
RESPESS James G,
LAIKIND Paul K,

CHANG Stephen M W,
CHADA Sunil D,
WARNER John F,
BARBER Jack R,
ST LOUIS Daniel C,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9102805 A2 19910307
Application: WO 90US4652 19900817 (PCT/WO US9004652)
Priority Application: US 89932 19890818; US 90606 19900810
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AT AU BE CA CH DE DK ES FI FR GB IT JP LU NL NO SE
Publication Language: English
Fulltext Word Count: 32135
? s s4 not s5
68 S4
50 S5
S6 18 S4 NOT S5
? t s6/6/all

6/6/1 (Item 1 from file: 155)
12080088 PMID: 9371630
Oligomerization-dependent folding of the membrane fusion protein of Semliki Forest virus.
Dec 1997

6/6/2 (Item 2 from file: 155)
11781041 PMID: 9116497
Purification and biochemical characterization of the 19-kDa signal recognition particle RNA-binding protein expressed as a hexahistidine-tagged polypeptide in Escherichia coli.
Feb 1997

6/6/3 (Item 3 from file: 155)
11022971 PMID: 7541239
Distinct biogenesis mechanisms for the water channels MIWC and CHIP28 at the endoplasmic reticulum.
Jul 4 1995

6/6/4 (Item 1 from file: 349)
00325766
CONJUGATES OF HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR WITH TARGETED AGENTS
CONJUGUÉS D'UN FACTEUR DE CROISSANCE SEMBLABLE AU FACTEUR DE CROISSANCE EPIDERMIQUE DE TYPE HBGF, ET D'AGENTS A CIBLE DEFINIE
Publication Language: English
Fulltext Availability:
Detailed Description
Claims
Fulltext Word Count: 58098
Publication Year: 1996

6/6/5 (Item 2 from file: 349)

00270158
ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF
PROTEINES DE FUSION DE LA TOXINE DU BACILLE DU CHARBON ET LEURS UTILISATIONS
Publication Language: English
Fulltext Availability:
  Detailed Description
  Claims
Fulltext Word Count: 42489
Publication Year: 1994

6/6/6 (Item 3 from file: 349)
00251391 **Image available**
RECOMBINANT PSEUDOMONAS EXOTOXIN WITH INCREASED ACTIVITY
EXOTOXINE DE PSEUDOMONAS RECOMBINEE A ACTIVITE ACCRUE
Publication Language: English
Fulltext Availability:
  Detailed Description
  Claims
Fulltext Word Count: 11944
Publication Year: 1993

6/6/7 (Item 4 from file: 349)
00209796
THERAPEUTIC FRAGMENTS OF VON WILLEBRAND FACTOR
FRAGMENTS THERAPEUTIQUES DU FACTOR DE VON WILLEBRAND
Publication Language: English
Fulltext Availability:
  Detailed Description
  Claims
Fulltext Word Count: 37055
Publication Year: 1992

6/6/8 (Item 1 from file: 654)
3897595
Derwent Accession: 1994-279753
Utility
C/ **Anthrax toxin fusion proteins and related methods**
; GENETIC ENGINEERING

Fulltext Word Count: 22109
Number of Claims: 12
Exemplary or Independent Claim Number(s): 1
Number of Drawing Sheets: 1
Number of Figures: 1
Number of non-patent cited references: 21

6/6/9 (Item 2 from file: 654)
3660828
Derwent Accession: 1995-373215
Utility
C/ **Expression of PACE in host cells and methods of use thereof**
; PAIRED BASIC AMINO ACID ENZYME

Fulltext Word Count: 21330
Number of Claims: 60
Exemplary or Independent Claim Number(s): 1
Number of Drawing Sheets: 12
Number of Figures: 2
Number of US cited patent references: 11
Number of non-US cited patent references: 3
Number of non-patent cited references: 46

6/6/10 (Item 3 from file: 654)
3174273 **IMAGE Available
Derwent Accession: 1990-107660
Utility
C/ **Novel pyrrolizidine alkaloid**
; INTERFERE WITH GLYCOPROTEIN PROCESSING IN INFLUENZA-INFECTED CELLS

Fulltext Word Count: 10622
Number of Claims: 9
Exemplary or Independent Claim Number(s): 1
Number of Drawing Sheets: 9
Number of Figures: 17
Number of US cited patent references: 3
Number of non-patent cited references: 27

6/6/11 (Item 1 from file: 34)
05464699 Genuine Article#: WA729 Number of References: 49
Title: FUNCTIONAL ANTIBODY-PRODUCTION USING CELL-FREE TRANSLATION - EFFECTS OF PROTEIN DISULFIDE-ISOMERASE AND CHAPERONES (Abstract Available)

6/6/12 (Item 2 from file: 34)
04570879 Genuine Article#: TU925 Number of References: 53
Title: A 2-STEP RECOGNITION OF SIGNAL SEQUENCES DETERMINES THE TRANSLOCATION EFFICIENCY OF PROTEINS (Abstract Available)

6/6/13 (Item 3 from file: 34)
02089045 Genuine Article#: KA059 Number of References: 73
Title: TARGETING SEQUENCES OF THE 2 MAJOR PEROXISOMAL PROTEINS IN THE METHYLOTROPHIC YEAST HANSENULA-POLYMORPHA (Abstract Available)

6/6/14 (Item 4 from file: 34)
01778998 Genuine Article#: JA763 Number of References: 48
Title: CORRECT FOLDING OF ALPHA-LYTIC PROTEASE IS REQUIRED FOR ITS EXTRACELLULAR SECRETION FROM ESCHERICHIA-COLI (Abstract Available)

6/6/15 (Item 1 from file: 5)
0011025227 BIOSIS NO.: 199799659287
Protein disulfide isomerase is the dominant acceptor for peptides translocated into the endoplasmic reticulum
1997

6/6/16 (Item 1 from file: 47)

03987152 SUPPLIER NUMBER: 14371235 (USE FORMAT 7 OR 9 FOR FULL TEXT)
Identification of a structural glycoprotein of an RNA virus as a ribonuclease.
August 27, 1993
WORD COUNT: 1540 LINE COUNT: 00134

6/6/17 (Item 1 from file: 434)
09564410 Genuine Article#: AC124 Number of References: 117
Title: MOLECULAR AND CELLULAR-REGULATION OF NEUROPEPTIDE EXPRESSION - THE BAG CELL MODEL SYSTEM

6/6/18 (Item 1 from file: 266)
00570672
IDENTIFYING NO.: 1R21AI060502-01A1 AGENCY CODE: CRISP
Role of antigen processing HIV CTL immunodominance
? logoff hold
08jul05 16:00:00 User228206 Session D2462.4
$0.20 0.060 DialUnits File155
$0.00 3 Type(s) in Format 6
$0.84 4 Type(s) in Format 9
$0.84 7 Types
$1.04 Estimated cost File155
$0.86 0.180 DialUnits File349
$1.60 1 Type(s) in Format 3
$1.00 4 Type(s) in Format 6
$2.60 5 Types
$3.46 Estimated cost File349
$0.81 0.137 DialUnits File654
$0.75 3 Type(s) in Format 6
$0.75 3 Types
$1.56 Estimated cost File654
$1.90 0.086 DialUnits File34
$0.00 4 Type(s) in Format 6
$6.43 1 Type(s) in Format 9
$6.43 5 Types
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$0.39 Estimated cost File440
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$0.00 1 Types
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$0.55 0.052 DialUnits File73
$0.55 Estimated cost File73
$0.51 0.094 DialUnits File348
$3.40 2 Type(s) in Format 3
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$3.91 Estimated cost File348
$0.15 0.017 DialUnits File71
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$0.46 0.086 DialUnits File47
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$6.90 2 Type(s) in Format 9
$6.90 3 Types
$7.36 Estimated cost File47
$0.05 0.009 DialUnits File156
$0.05 Estimated cost File156

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$0.04   Estimated cost File149
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$0.08   Estimated cost File484
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$0.16   Estimated cost File8
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        $0.00  1 Types
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        OneSearch, 30 files,  1.193 DialUnits FileOS
$0.26   TELNET
$34.36  Estimated cost this search
$34.36  Estimated total session cost   1.193 DialUnits

Logoff: level 05.05.00 D  16:00:00

You are now logged off
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04644957 Genuine Article#: TZ983 Number of References: 132
Title: COMMON PRINCIPLES OF PROTEIN TRANSLOCATION ACROSS MEMBRANES
Author(s): SCHATZ G; DOBBERSTEIN B
Corporate Source: UNIV BASEL,BIOCTR,KLINGELBERGSTR 70/CH-4056 BASEL//SWITZERLAND/; UNIV HEIDELBERG,ZENTRUM MOLEK BIOL/D-69120 HEIDELBERG//GERMANY/
Journal: SCIENCE, 1996, V271, N5255 (MAR 15), P1519-1526
ISSN: 0036-8075
Language: ENGLISH Document Type: REVIEW
Geographic Location: SWITZERLAND; GERMANY
Subfile: SciSearch; CC PHYS--Current Contents, Physical, Chemical & Earth Sciences; CC LIFE--Current Contents, Life Sciences; CC AGRI--Current Contents, Agriculture, Biology & Environmental Sciences
Journal Subject Category: MULTIDISCIPLINARY SCIENCES
Abstract: Most major systems that transport proteins across a membrane share the following features: an amino-terminal transient signal sequence on the transported protein, a targeting system on the cis side of the membrane, a hetero-oligomeric transmembrane channel that is gated both across and within the plane of the membrane, a peripherally attached protein translocation motor that is powered by the hydrolysis of nucleoside triphosphate, and a protein folding system on the trans side of the membrane. These transport systems are divided into two families: export systems that export proteins out of the cytosol, and import systems that transport proteins into cytosol-like compartments.
Identifiers--KeyWords Plus: REQUIRES NUCLEOSIDE TRIPHOSPHATES; MITOCHONDRIAL INNER MEMBRANE; IMMUNOGLOBULIN LIGHT-CHAINS; SIGNAL RECOGNITION PARTICLE; ESCHERICHIA-COLI; ENDOPLASMIC-RETICULUM; YEAST MITOCHONDRIA; INTERMEMBRANE SPACE; PRECURSOR PROTEINS; THYLAKOID MEMBRANE
Research Fronts: 94-1621 001 (MITOCHONDRIAL PROCESSING PEPTIDASE; PROTEIN IMPORT; PREPROTEINS OF CHLOROPLAST ENVELOPE INNER MEMBRANE CONTAIN TARGETING INFORMATION)
94-1770 001 (PROTEIN **DISULFIDE** BOND FORMATION; HUMAN PLACENTAL ALKALINE-PHOSPHATASE IN ESCHERICHIA-CO

(c) 2005 INIST/CNRS
File 370:Science 1996-1999/Jul W3
(c) 1999 AAAS
*File 370: This file is closed (no updates). Use File 47 for more current information.
File 636:Gale Group Newsletter DB(TM) 1987-2005/Jul 07
(c) 2005 The Gale Group
File 16:Gale Group PROMT(R) 1990-2005/Jul 07
(c) 2005 The Gale Group
File 50:CAB Abstracts 1972-2005/Jun
(c) 2005 CAB International
File 162:Global Health 1983-2005/Jun
(c) 2005 CAB International
File 10:AGRICOLA 70-2005/Jun
(c) format only 2005 The Dialog Corporation
File 135:NewsRx Weekly Reports 1995-2005/Jul W1
(c) 2005 NewsRx
*File 135: New newsletters are now added. See Help News135 for the complete list of newsletters.
File 172:EMBASE Alert 2005/Jul 08
(c) 2005 Elsevier Science B.V.
File 266:FEDRIP 2005/Jun
Comp & dist by NTIS, Intl Copyright All Rights Res
File 315:ChemEng & Biotec Abs 1970-2005/Jun
(c) 2005 DECHEMA

Set	Items	Description

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? s
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? t s6/3,kwic/8 6 5 4

6/3,KWIC/8 (Item 1 from file: 654)
DIALOG(R)File 654:US Pat.Full.
(c) Format only 2005 The Dialog Corp. All rts. reserv.

3897595
Derwent Accession: 1994-279753
Utility
CERTIFICATE OF CORRECTION
C/ Anthrax toxin fusion proteins and related methods
; GENETIC ENGINEERING
Inventor: Leppla, Stephen H., Bethesda, MD
Klimpel, Kurt R., Gaithersburg, MD
Arora, Naveen, Delhi, IN
Singh, Yogendra, Delhi, IN
Nichols, Peter J., Welling Kent, GB
Assignee: The Government of the United States as represented by the Secretary of the Department of Health and Human Services(06), Washington, DC
U S of America Health & Human Services (Code: 06814)
Examiner: Jagannathan, Vasu S. (Art Unit: 181)
Assistant Examiner: Romeo, David S.
Law Firm: Townsend and Townsend and Crew

	Publication Number	Kind	Date	Application Number	Filing Date
Main Patent	US 5677274	A	19971014	US 9382849	19930625

CIP US 5591631 A US 9321601 19930212

Fulltext Word Count: 22109

Summary of the Invention:
...need exists for a means to target molecules having a desired activity to a specific cell population...

...of mammalian cells, and this ability has been exploited to develop anti-tumor and anti- HIV cytotoxic agents. Examples include ricin and Pseudomonas exotoxin A (PE) chimeric toxins and immunotoxins...

...receptor-binding domain (residues 1-252 and 365-399) designated Ia and Ib, a central translocation domain (amino acids 253-364, domain II), and a carboxyl-terminal enzymatic domain (amino acids...

...ribosylation of elongation factor 2 (EF-2), which results in inhibition of protein synthesis and cell death. Recently it was also found that an extreme carboxyl terminal sequence is essential for in the endoplasmic reticulum (ER) (Munro, S. and Pelham, H. R. B. Cell 48:899-907, 1987), it was suggested that PE must pass through the ER to...

6/3,KWIC/6 (Item 3 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00251391 **Image available**
RECOMBINANT PSEUDOMONAS EXOTOXIN WITH INCREASED ACTIVITY
EXOTOXINE DE PSEUDOMONAS RECOMBINEE A ACTIVITE ACCRUE
Patent Applicant/Assignee:
THE UNITED STATES OF AMERICA as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES,
Inventor(s):
PASTAN Ira H,
FITZGERALD David J,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9325690 A1 19931223
Application: WO 93US5858 19930617 (PCT/WO US9305858)
Priority Application: US 92901709 19920618
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AU CA JP AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE
Publication Language: English
Fulltext Word Count: 11944

Fulltext Availability:
Detailed Description

Detailed Description
... of
domain II results in increased toxic activity since this domain is responsible for the translocation of the toxin into the cytosol,
In addition, the PE molecules can be further modified...

...which

have been found to be effective include, REDLK (as in native PE), REDL or KDEL , repeats of those, or other sequences that function to maintain or recycle proteins into the endoplasmic reticulum , referred to here as Ilendoplasmic retention sequences". See, for example, Chaudhary et al, Rroc. Natl...

...serine at amino acid position 287 in place of cysteine to prevent formation of improper disulfide bonds is beneficial.

A I'ligand ...capable of reacting with or otherwise recognizing or binding to a receptor on a target cell . Examples of such binding agents include, but are not limited to, antibodies, growth factors such...

6/3,KWIC/5 (Item 2 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00270158
ANTHRAX TOXIN FUSION PROTEINS AND USES THEREOF
PROTEINES DE FUSION DE LA TOXINE DU BACILLE DU CHARBON ET LEURS UTILISATIONS
Patent Applicant/Assignee:
THE GOVERNMENT OF THE UNITED STATES OF AMERICA as represented by THE SECRETARY OF THE DEPARTMENT OF HEALTH AND HUMAN SERVICES,
Inventor(s):
LEPPLA Stephen H,
KLIMPEL Kurt,
ARORA Naveen,
SINGH Yogendra,
NICHOLS Peter J,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9418332 A2 19940818
Application: WO 94US1624 19940214 (PCT/WO US9401624)
Priority Application: US 9321601 19930212; US 9382849 19930625
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AU CA JP AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE
Publication Language: English
Fulltext Word Count: 42489

Fulltext Availability:
Detailed Description

Detailed Description
... approach also has inherent disadvantages, because it requires availability of the parent hybridoma or myeloma cell line, and problems are often encountered when attempting to retrieve the correct V region genes...need exists for a means to target molecules having a desired activity to a specific cell population.

Bacterial and plant protein toxins have evolved novel and efficient strategies for penetrating to...

...of mammalian cells, and this ability has been exploited to develop anti-tumor and anti- HIV cytotoxic agents. Examples include ricin and Pseudomonas exotoxin A (PE) chimeric toxins

and immunotoxins.

Pseudomonas...
...receptor-binding domain (residues 1-252 and 365-399), designated Ia and Ib, a central translocation domain (amino acids 253-364, domain II), and a carboxyl-terminal enzymatic domain (amino acids...

...ribosylation of elongation factor 2 (EF-2), which results in inhibition of protein synthesis and cell death. Recently it was also found that an extreme carboxyl terminal sequence is essential for...

...this sequence is similar to the sequence that specifies retention of proteins in the endoplasmic reticulum (ER) '(Munro, S. and Pelham, H.R.B. Cell 48:899-907, 1987), it was suggested that PE must pass through the ER to...ET and LT each conform to the AB toxin model, with PA providing the target cell binding (B) function and EF or LF acting as the effector or catalytic (A) moieties...

6/3,KWIC/4 (Item 1 from file: 349)
DIALOG(R)File 349:PCT FULLTEXT

00325766
CONJUGATES OF HEPARIN-BINDING EPIDERMAL GROWTH FACTOR-LIKE GROWTH FACTOR WITH TARGETED AGENTS
CONJUGUES D'UN FACTEUR DE CROISSANCE SEMBLABLE AU FACTEUR DE CROISSANCE EPIDERMIQUE DE TYPE HBGF, ET D'AGENTS A CIBLE DEFINIE
Patent Applicant/Assignee:
PRIZM PHARMACEUTICALS INC,
Inventor(s):
SOSNOWSKI Barbara A,
CHANDLER Lois Ann,
HOUSTON L L,
NOVA Michael P,
McDONALD John R,
Patent and Priority Information (Country, Number, Date):
Patent: WO 9608274 A2 19960321
Application: WO 95US12205 19950913 (PCT/WO US9512205)
Priority Application: US 94305771 19940913; US 95441979 19950516
Designated States:
(Protection type is "patent" unless otherwise stated - for applications prior to 2004)
AM AU BB BG BR BY CA CN CZ EE FI GE HU IS JP KG KP KR KZ LK LR LT LV MD MG MN MX NO NZ PL RO RU SG SI SK TJ TM TT UA UZ VN KE MW SD SZ UG AT BE CH DE DK ES FR GB GR IE IT LU MC NL PT SE BF BJ CF CG CI CM GA GN ML MR NE SN TD TG
Publication Language: English
Fulltext Word Count: 58098

Fulltext Availability:
Detailed Description

Detailed Description
... internalized ligand/nucleic acid binding domain conjugate or fusion to deliver nucleic acids into a cell .

Other elements
11 Nuclear translocation signals
As used herein, a nuclear translocation or targeting sequence...

...amino acids in a protein that are required for translocation of the protein into a cell nucleus. Examples of NTS are set forth in Table 2, below. Comparison with known NTSs...

...Dang et al. (1989) J Biol.
Chem. 264:18019-18023, Dang et al. (1988) Mol. Cell . Biol. 8:4049-4058 and Table 2, which sets forth examples of NTSs and regions...
LeuLeuLysLyslleLvsGln 75
3!6
p5-1 Pro GlnProLysLvsLysPro 76
277
Nucleolin Pro GlyLysArgLvsLysGIuMetThrLvsGinLvsGIuValPro 77
48
HIV Tat Gly ArgLysLysArgArgGInArgArgArgAlaPro 78
FGF- I AsnTyrLysLysProLysLeu 79
FGF-2 HisPheLysAspProLysArg 80
FGF-3 AlaProArgArgArgLysLeu...

...VEGF206 LysArgLysArgLysLys (in EXON VI) 66
PDGF ProLysGlyLysHisArgLysPheLysHisThI
*Superscript indicates position in protein
21 Cytoplasm- translocation signal
Cytoplasm- translocation signal sequence is a sequence of amino acids in a protein that cause retention of proteins in the lumen of the endoplasmic reticulum and/or translocate proteins to the cytosol. The signal sequence in mammalian cells is KDFL (Lys-Asn-Cilti-Leu) (Munro and Pelham, Cell 48:899-907, 1987). SoAme modification s of this sequence have been made without loss of activity. For example, the sequences RDEL (Arg-Asp-Glu-Leu) and KEEL (Lys-Glu-Glu-Leu) confer efficient or partial retention, respectively, in plants (Denecke et al., Embo. J 11:2345-2355, 1992).

A cytoplasm- translocation signal sequence may be included in saporin or, for conjugates of HBEGF with a nucleic...

...signal is preferably included in saporin or the nucleic acid binding domain. Additionally, a cytoplasmic- translocation signal sequence may be included in HBEGF, as long as it is placed so as...
? t s6/9/1 18

6/9/1 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12080088 PMID: 9371630
Oligomerization-dependent folding of the membrane fusion protein of Semliki Forest virus.
Andersson H; Barth B U; Ekstrom M; Garoff H
Department of Biosciences at Novum, Huddinge, Sweden.
Journal of virology (UNITED STATES) Dec 1997, 71 (12) p9654-63, ISSN 0022-538X Journal Code: 0113724
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM

Record type: MEDLINE; Completed
Subfile: INDEX MEDICUS

The spikes of alphaviruses are composed of three copies of an E2-E1 heterodimer. The E1 protein possesses membrane fusion activity, and the E2 protein, or its precursor form, p62 (sometimes called PE2), controls this function. Both proteins are, together with the viral capsid protein, translated from a common C-p62-E1 coding unit. In an earlier study, we showed that the p62 protein of Semliki Forest virus (SFV) dimerizes rapidly and efficiently in the endoplasmic reticulum (ER) with the E1 protein originating from the same translation product (so-called heterodimerization in cis) (B.-U. Barth, J. M. Wahlberg, and H. Garoff, J. Cell Biol. 128:283-291, 1995). In the present work, we analyzed the ER translocation and folding efficiencies of the p62 and E1 proteins of SFV expressed from separate coding units versus a common one. We found that the separately expressed p62 protein translocated and folded almost as efficiently as when it was expressed from a common coding unit, whereas the independently expressed E1 protein was inefficient in both processes. In particular, we found that the majority of the translocated E1 chains were engaged in disulfide-linked aggregates. This result suggests that the E1 protein needs to form a complex with p62 to avoid aggregation. Further analyses of the E1 aggregation showed that it occurred very rapidly after E1 synthesis and could not be avoided significantly by the coexpression of an excess of p62 from a separate coding unit. These latter results suggest that the p62-E1 heterodimerization has to occur very soon after E1 synthesis and that this is possible only in a cis-directed reaction which follows the synthesis of p62 and E1 from a common coding unit. We propose that the p62 protein, whose synthesis precedes that of the E1 protein, remains in the translocon of the ER and awaits the completion of E1. This strategy enables the p62 protein to complex with the E1 protein immediately after the latter has been made and thereby to control (suppress) its fusion activity.

Tags: Research Support, Non-U.S. Gov't
Descriptors: *Protein Folding; *Semliki forest virus--metabolism--ME; *Viral Envelope Proteins--biosynthesis--BI; Animals; Endoplasmic Reticulum --metabolism--ME; Gene Expression; Hamsters; RNA, Viral; Semliki forest virus--genetics--GE; Sequence Deletion; Transfection; Viral Envelope Proteins--genetics--GE
CAS Registry No.: 0 (RNA, Viral); 0 (Viral Envelope Proteins)
Record Date Created: 19971224
Record Date Completed: 19971224

6/9/18 (Item 1 from file: 266)
DIALOG(R)File 266:FEDRIP

00570672
IDENTIFYING NO.: 1R21AI060502-01A1 AGENCY CODE: CRISP
Role of antigen processing HIV CTL immunodominance
PRINCIPAL INVESTIGATOR: LEGALL, SYLVIE J
ADDRESS: SYLVIE LEGALL@HMS.HARVARD.EDU MASSACHUSETTS GENERAL HOSPITAL 149 13TH ST, RM 6624 CHARLESTOWN, MA 02129
PERFORMING ORG.: MASSACHUSETTS GENERAL HOSPITAL, BOSTON, MASSACHUSETTS
SPONSORING ORG.: NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
DATES: 2007/01/04 TO 2006/30/06 FY : 2004 TYPE OF AWARD: New Award (Type 1)
SUMMARY: DESCRIPTION (provided by applicant): Cytotoxic T-lymphocytes (CTL) are essential for immune control in HIV infection, and immunodominant CTL responses have been characterized for many HLA alleles. However the reasons for relative immunodominance of certain epitopes have not been defined, and the steps in antigen processing that shape an immunodominant

epitope are not known. Moreover no studies have examined how HLA-restricted mutations that accumulate in HIV-1 infected people affect the generation of epitopes. Our long-term goal is to understand the rules that govern degradation and presentation of specific HIV antigens and the impairment of antigen presentation through mutations. In preliminary studies, we have identified allele-specific mutations in the flanking regions of dominant HIV epitopes that impair antigen processing and recognition by CTL. We have shown that an HLA-restricted mutation can affect trimming of longer peptide precursors in the endoplasmic reticulum (ER), thereby altering the relative availability of epitopes for class I loading. We hypothesize that antigen processing influences the hierarchy observed among epitopes and that specific HLA-associated mutations in flanking regions of epitopes alter distinct steps of the processing and serve as a mechanism for immune escape. This proposal will focus on a detailed analysis of changes in antigen processing pathways that may lead to viral escape. The specific aims include: 1. To define cytosolic processing ofCD8 T cell epitopes, focusing on HLA-A3/A11-restricted responses for initial studies; 2. To determine the extent to which N-glycosylation of peptides in the ER serves as a potential mechanism of viral escape from CTL responses. These goals are based on a common experimental design. We will follow the complete processing of HIV epitopes of interest in antigen presenting cells or reconstruct this process with subcellular fractions. We will analyze the degradation of HIV polypeptides in the cytosol by the proteasome and other peptidases, the translocation into the ER and the trimming and potential N-glycosylation of N-extended peptides in the ER lumen, the loading of peptides onto MHC-I molecules and their subsequent presentation to CTL. Given the extreme mutation rate of HIV and the persistence of immune selection pressure, these studies represent a critical first step in determining the role of antigen processing in immune escape, which will be important not only for the understanding of HIV immunopathogenesis but also for the design of candidate vaccines.

DESCRIPTORS: cytotoxic T lymphocyte; endoplasmic reticulum; polymerase chain reaction; electroporation; host organism interaction; gene mutation; glycosylation; human tissue; cellular immunity; western blotting; antigen presentation; virus antigen; immunopathology; AIDS; HIV infection; high performance liquid chromatography; protein transport; virus replication; green fluorescent protein; immune response

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☐ 1. Document ID: US 20050079171 A1

L13: Entry 1 of 32 File: PGPB Apr 14, 2005

DOCUMENT-IDENTIFIER: US 20050079171 A1
TITLE: Pseudomonas exotoxin A-like chimeric immunogens for eliciting a secretory IgA-mediated immune response

CLAIMS:

1. A method of eliciting a secretory IgA-mediated immune response in a subject comprising the step of administering to at least one mucosal surface of the subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a foreign epitope; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

11. The composition of claim 8 produced by administering to at least one mucosal surface of a subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a an epitope of HIV-1; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw D

☐ 2. Document ID: US 20050033033 A1

L13: Entry 2 of 32 File: PGPB Feb 10, 2005

DOCUMENT-IDENTIFIER: US 20050033033 A1
TITLE: Trans-membrane-antibody induced inhibition of apoptosis

CLAIMS:

1. A compound effective in regulating normal or infected cell function, which compound comprises an antibody, or fragment thereof, conjugated to a membrane transporter peptide, which antibody, or fragment thereof, is immmunospecific for: (a) a signaling protein internal a cell selected from the group consisting of caspases, kinases, and phosphatases, (b) an immature viral protein, (c) a cell-surface or intracellular tumor antigen, (d) a nuclear or nucleolar protein participating in regulation of DNA synthesis and gene expression, or (e) a cytoskeletal protein participating in cell proliferation or cytostasis.

5. The compound of claim 1, wherein the membrane transporter peptide is a translocation sequence (MTS) peptide.

13. The composition of claim 10, wherein the membrane transporter peptide is a membrane translocation sequence (MTS) peptide.

21. The immunoconjugate of claim 18, wherein the membrane transporter peptide, or fragment thereof, is covalently linked to a tryptophan residue or nucleotide binding site of the secondary antibody.

23. A method of treating or preventing disease in humans comprising: pre-administering a primary antibody, immunospecific for a cell-surface target, to a patient in need thereof; allowing sufficient time for binding of the primary antibody to the target and clearance from normal tissues; and administering a secondary antibody covalently linked to a membrane transporter peptide or fragment thereof, which secondary antibody is immunospecific for the primary antibody.

25. The method of claim 24, wherein the toxin is (a) a holo-protein toxin, selected from the group consisting of ricin, abrin, diphtheria and Pseudomonas exotoxins, (b) a whole protein toxin subunit, or (b) a naturally-occurring A-chain toxin subunit selected from the group consisting of ricin A chain, abrin A chain, diphtheria toxin A chain, Pseudomonas exotoxin A and gelonin.

30. The method of claim 29, wherein the toxin is (a) a holo-protein toxin, selected from the group consisting of ricin, abrin, diphtheria and Pseudomonas exotoxins, (b) a whole protein toxin subunit, or (b) a naturally-occurring A-chain toxin subunit selected from the group consisting of ricin A chain, abrin A chain, diphtheria toxin A chain, Pseudomonas exotoxin A and gelonin.

35. An in-vitro screen assay comprising: contacting a primary antibody immunospecific for a cellular receptor or intracellular target with a plurality of cells, wherein the primary antibody is conjugated with a membrane transporter peptide or fragment thereof; and assessing a potential for antagonism or agonism of cellular activity due to internalization of the primary antibody.

36. An in-vitro screen assay comprising: contacting a primary antibody immunospecific for a cellular receptor or intracellular target with a plurality of cells; admixing a secondary antibody conjugated to a membrane transporter peptide, or fragment thereof, with the primary antibody, which secondary antibody is immunospecific for the primary antibody; and assessing a potential for antagonism or agonism of cellular activity due to internalization of the primary antibody.

3. Document ID: US 20040247617 A1

L13: Entry 3 of 32 File: PGPB Dec 9, 2004

DOCUMENT-IDENTIFIER: US 20040247617 A1
TITLE: Fusion antigen used as vaccine

CLAIMS:

1. A fusion antigen specific for a target cell comprising: an antigenic moiety; a ligand moiety which is capable of reacting, recognizing or binding to a receptor on the target cell; a Pseudomonas exotoxin A translocation domain II; and a carboxyl terminal moiety which permits retention of the fusion antigen in the endoplasmic reticulum (ER) membrane of the target cell.

2. The fusion antigen according to claim 1, wherein the target cell is an antigen presenting cell.

3. The fusion antigen according to claim 1, wherein the target cell is selected from the group consisting of T-cells, B-cells, dendritic cells, monocytes, and macrophages.

7. The fusion antigen according to claim 1, wherein the receptor to be bound to the ligand moiety is selected from the group consisting of antibody receptors, growth factor receptors, lymphokine receptors, cytokine receptors, and hormone receptors.

8. The fusion antigen according to claim 1, wherein the receptor to be bound to the ligand moiety is selected from the group consisting of TGF.alpha. receptors, IL2 receptors, IL4 receptors, IL6 receptors, IGF 1 receptors, CD4 receptors, IL18 receptors, IL 12 receptors, EGF receptors, LDL receptors and .alpha.2-macroglobulin receptors.

9. The fusion antigen according to claim 1, wherein the ligand moiety is a Pseudomonas exotoxin A binding domain I.

14. The pharmaceutical composition according to claim 13 is a T-cell vaccine.

15. A method of immunizing an animal comprising the steps of: (a) providing a fusion antigen specific for a target cell comprising an antigenic moiety, a ligand moiety which is capable of reacting, recognizing or binding to a receptor on the target cell, a Pseudomonas exotoxin A translocation domain II, and a carboxyl terminal moiety which permits retention of the fusion antigen in the endoplasmic reticulum (ER) membrane of the target cell; and (b) inoculating the fusion antigen into the animal.

16. The method according to claim 15, wherein the target cell is an antigen presenting cell.

20. The method according to claim 15, wherein the ligand moiety is a Pseudomonas exotoxin A binding domain I.

21. The method according to claim 15, wherein the receptor to be bound to the ligand moiety is selected from the group consisting of antibody receptors, growth factor receptors, lymphokine receptors, cytokine receptors, and hormone receptors.

22. The method according to claim 15, wherein the receptor to be bound to the ligand moiety is selected from the group consisting of TGF.alpha. receptors, IL2 receptors, IL4 receptors, IL6 receptors, IGF 1 receptors, CD4 receptors, IL18 receptors, IL 12 receptors, EGF receptors, LDL receptors and .alpha.2-macroglobulin receptors.

23. The method according to claim 15, wherein the target cell is selected from the

group consisting of T cell, B cell, dendritic cell, monocyte, and macrophage.

27. A fusion porcine reproductive and respiratory syndrome virus (PRRSV) ORF 7 antigen comprising a PRRSV ORF 7 moiety; a Pseudomonas exotoxin A binding domain I; a Pseudomonas exotoxin A translocation domain II; and a carboxyl terminal moiety which permits retention of the fusion antigen in the endoplasmic reticulum (ER) membrane of a target cell.

28. The fusion antigen according to claim 27, wherein the target cell is an antigen presenting cell.

29. The fusion antigen according to claim 27, wherein the target cell is selected from the group consisting of T cell, B cell, dendritic cell, monocyte, and macrophage.

35. The pharmaceutical composition according to claim 34 is a T-cell vaccine.

36. A method of immunizing an animal for the preventing porcine reproductive and respiratory syndrome virus (PRRSV), which comprises the steps of: (a) providing a fusion antigen comprising a PRRSV ORF 7 antigen moiety, a Pseudomonas exotoxin A binding domain I, a Pseudomonas exotoxin A translocation domain II, and a carboxyl terminal moiety which permits retention of the antigen in the endoplasmic reticulum (ER) membrane of a target cell; and (b) inoculating the fusion antigen into the animal.

37. The method according to claim 36, wherein the target cell is an antigen presenting cell.

38. The method according to claim 36, wherein the target cell is selected from the group consisting of T-cells, B-cells, dendritic cells, monocytes, and macrophages.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw D

☐ 4. Document ID: US 20040191260 A1

L13: Entry 4 of 32 File: PGPB Sep 30, 2004

DOCUMENT-IDENTIFIER: US 20040191260 A1
TITLE: Compositions capable of specifically binding particular human antigen presenting molecule/pathogen-derived antigen complexes and uses thereof

CLAIMS:

1. A composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

5. The composition-of-matter of claim 1, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

8. The composition-of-matter of claim 1, wherein said binding of said antibody or

antibody fragment to said antigen-presenting portion of said complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

14. The composition-of-matter of claim 9, wherein said toxin is Pseudomonas exotoxin A or a portion thereof.

15. The composition-of-matter of claim 14, wherein said portion of Pseudomonas exotoxin A is a translocation domain and/or an ADP ribosylation domain.

27. A composition-of-matter comprising a multimeric form of an antibody or antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

31. The composition-of-matter of claim 27, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14to 97.

34. The composition-of-matter of claim 27, wherein said binding of said antibody or antibody fragment to said antigen-presenting portion of said complex is characterized by a binding affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

40. The composition-of-matter of claim 35, wherein said toxin is Pseudomonas exotoxin A or a portion thereof.

41. The composition-of-matter of claim 40, wherein said portion of Pseudomonas exotoxin A is a translocation domain and/or an ADP ribosylation domain.

53. An isolated polynucleotide comprising a nucleic acid sequence encoding an antibody fragment, said antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

60. The isolated polynucleotide of claim 54, wherein said toxin is Pseudomonas exotoxin A or a portion thereof.

61. The isolated polynucleotide of claim 60, wherein said portion of Pseudomonas exotoxin A is a translocation domain and/or an ADP ribosylation domain.

62. The isolated polynucleotide of claim 53, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

65. The isolated polynucleotide of claim 53, wherein said binding of said antibody fragment to said antigen-presenting portion of said complex is characterized by a binding affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.16 molar.

76. A nucleic acid construct comprising the isolated polynucleotide of claim 53 and a promoter sequence for directing transcription of the isolated polynucleotide in a host cell.

80. A host cell comprising the nucleic acid construct of claim 76.

81. The host cell of claim 80, wherein the host cell is a prokaryotic cell.

82. The host cell of claim 81, wherein said prokaryotic cell is an E. coli cell.

85. A virus comprising a coat protein fused to an antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

88. The virus of claim 85, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

89. The virus of claim 85, wherein said binding of said antibody fragment to said antigen-presenting portion of said complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

101. A method of detecting an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen, the method comprising: (a) exposing the antigen-presenting portion of the complex to a composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding the antigen-presenting portion of the complex, to thereby obtain a -conjugate of the antigen-presenting portion of the complex and said antibody or antibody fragment; and (b) detecting said antibody or antibody fragment of said conjugate, thereby detecting an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

102. The method of claim 101, wherein the complex is displayed or expressed by a target cell, and whereas step (a) is effected by exposing said target cell to said composition-of-matter.

103. The method of claim 102, further comprising: (c) obtaining said target cell from an individual.

104. The method of claim 102, wherein said exposing said target cell to said composition-of-matter is effected by administering said composition-of-matter to an individual.

105. The method of claim 102, wherein said target cell is a T lymphocyte or an antigen presenting cell.

106. The method of claim 105, wherein said antigen presenting cell is a B cell or a dendritic cell.

110. The method of claim 101, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

111. The method of claim 101, wherein said binding of said antibody or antibody fragment to the antigen-presenting portion of the complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

122. A method of diagnosing an infection by a pathogen in an individual, the method comprising: (a) exposing a target cell of the individual to a composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from the pathogen, to thereby obtain a conjugate of said antigen-presenting portion of said complex and said antibody or antibody fragment; and (b) detecting said antibody or antibody fragment of said conjugate, thereby diagnosing an infection by a pathogen in an individual.

123. The method of claim 122, further comprising: (c) obtaining said target cell

from the individual.

125. The method of claim 122, wherein said target cell is a T lymphocyte or an antigen presenting cell.

126. The method of claim 122, wherein said antigen presenting cell is a B cell or a dendritic cell.

130. The method of claim 122, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

131. The method of claim 122, wherein said binding of said antibody or antibody fragment to the antigen-presenting portion of the complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

141. A method of killing or damaging a target cell expressing or displaying an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen, the method comprising exposing the target cell to a composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding the antigen-presenting portion of the complex, thereby killing or damaging a target cell expressing or displaying an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from a pathogen.

143. The method of claim 142, wherein said toxin is Pseudomonas exotoxin A or a portion thereof.

144. The method of claim 141, further comprising the step of obtaining the target cell from an individual.

145. The method of claim 141, wherein said exposing the cell to said composition-of-matter is effected by administering said composition-of-matter to an individual.

146. The method of claim 141, wherein the target cell is infected with the pathogen.

147. The method of claim 141, wherein the target cell is a T lymphocyte or an antigen presenting cell.

148. The method of claim 141, wherein said antigen presenting cell is a B cell or a dendritic cell.

150. The method of claim 141, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

151. The method of claim 141, wherein said binding of said antibody or antibody fragment to said antigen-presenting portion of said complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

161. A method of treating a disease associated with a pathogen in an individual, the method comprising administering to the individual a therapeutically effective amount of a pharmaceutical composition comprising as an active ingredient, a composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding an antigen-presenting portion of a complex composed of a human antigen-presenting molecule and an antigen derived from the pathogen, thereby treating a disease associated with a pathogen in an individual.

163. The method of claim 162, wherein said toxin is Pseudomonas exotoxin A or a portion thereof.

165. The method of claim 161, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

166. The method of claim 161, wherein said binding of said antibody or antibody fragment to said antigen-presenting portion of said complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-16 molar.

176. A method of detecting in a biological sample an antigen-presenting portion of a complex composed of an antigen-presenting molecule and an antigen, the method comprising: (a) attaching the biological sample to a surface; (b) exposing the biological sample to a composition-of-matter comprising an antibody or antibody fragment including an antigen-binding region capable of specifically binding the antigen-presenting portion of the complex, to thereby obtain a conjugate of the antigen-presenting portion of the complex and said antibody or antibody fragment; and (c) detecting said antibody or antibody fragment of said conjugate, thereby detecting in a biological sample an antigen-presenting portion of a complex composed of an antigen-presenting molecule and an antigen.

186. The method of claim 176, wherein the biological sample is a cell sample or a tissue sample.

188. The method of claim 176, wherein said antigen-binding region includes an amino acid sequence selected from the group consisting of SEQ ID NOs: 14 to 97.

189. The method of claim 176, wherein said binding of said antibody or antibody fragment to the antigen-presenting portion of the complex is characterized by an affinity having a dissociation constant selected from the range consisting of 1.times.10.sup.-2 molar to 5.times.10.sup.-6 molar.

☐ 5. Document ID: US 20040086845 A1

L13: Entry 5 of 32 File: PGPB May 6, 2004

DOCUMENT-IDENTIFIER: US 20040086845 A1
TITLE: Superior molecular vaccine linking the translocation domain of a bacterial toxin to an antigen

CLAIMS:

1. A nucleic acid encoding a chimeric or fusion polypeptide which polypeptide comprises: (a) a first domain comprising a translocation polypeptide; and (b) a second domain comprising at least one antigenic peptide.

2. The nucleic acid of claim 2 wherein the translocation polypeptide is a bacterial toxin translocation polypeptide.

3. The nucleic acid of claim 2 wherein the translocation bacterial toxin translocation polypeptide is Pseudomonas aeruginosa exotoxin A domain II (ETA (dII)).

4. The nucleic acid of claim 1 wherein the translocation polypeptide is SEQ ID NO:3 or a homologue thereof.

5. The nucleic acid of claim 1 that comprises a nucleotide sequence that encodes a translocation polypeptide which sequence is included in SEQ ID NO:1.

6 The nucleic acid of claim 1, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins.

7 The nucleic acid of claim 6 wherein the epitope is between about 8 and about 11 amino acid residues in length.

8 The nucleic acid of claim 1, wherein the antigen (i) is derived from a pathogen selected from the group consisting of a mammalian cell, a microorganism or a virus; or (ii) cross-reacts with an antigen of the pathogen.

15. The nucleic acid of claim 14, wherein the promoter is one which is expressed in an antigen presenting cell (APC).

16. The nucleic acid of claim 15, wherein the APC is a dendritic cell.

17. An expression vector comprising (a) the nucleic acid of any of claims 1-16 operatively linked to (b) a promoter and, optionally, to one or more regulatory elements that enhance expression of said nucleic acid in a cell.

20. The expression vector of claim 17 wherein the translocation polypeptide is ETA (dII).

23. The particle of claim 21 which comprises a material is suitable for introduction into a cell or an animals by particle bombardment.

25. A cell which has been modified to comprise the nucleic acid of any of claims 1-16.

26. A cell which has been modified to comprise the expression vector of claim 17, and which cell expresses said nucleic acid.

27. The cell of claim 26 which is an APC.

28. The cell of claim 27, wherein the APC is a dendritic cell, a keratinocyte, a macrophage, a monocyte, a B lymphocyte, a microglial cell, an astrocyte, or an activated endothelial cell.

29. A chimeric polypeptide comprising (a) a first domain comprising a translocation polypeptide; and (b) a second domain comprising at least one antigenic peptide.

30. The chimeric polypeptide of claim 29 wherein the translocation polypeptide is a bacterial toxin translocation polypeptide.

31. The chimeric polypeptide of claim 30 wherein the translocation bacterial toxin translocation polypeptide is Pseudomonas aeruginosa exotoxin A domain II (ETA (dII)).

32. The chimeric polypeptide of claim 29 wherein the translocation polypeptide is SEQ ID NO:3 or a homologue thereof.

34. The chimeric polypeptide of any of claims claim 29-32, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins.

35. The chimeric polypeptide of claim 33, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins.

36. The chimeric polypeptide of any of claims 29-32 wherein the translocation domain and the antigenic peptide are linked by a chemical linker.

43. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 25, and a pharmaceutically acceptable carrier or excipient.

44. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of particle of claim 26, and a pharmaceutically acceptable carrier or excipient.

45. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 27, and a pharmaceutically acceptable carrier or excipient.

46. A pharmaceutical composition capable of inducing or enhancing an antigen specific immune response, comprising the cell of claim 28, and a pharmaceutically acceptable carrier or excipient.

52. A method of inducing or enhancing an antigen specific immune response comprising administering an effective amount of a composition comprising the cell of claim 25, thereby inducing or enhancing the antigen specific immune response.

62. The method of claim 61, wherein the APCs are dendritic cells.

70. A method of increasing the numbers of CD8.sup.+ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising the nucleic acid molecule of any of claims 1-16, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific CD8.sup.+ CTLs.

71. A method of increasing the numbers of CD8.sup.+ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising the expression vector of claim 17, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific CD8.sup.+ CTLs.

72. A method of increasing the numbers of CD8.sup.+ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising the particle of claim 21, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific CD8.sup.+ CTLs.

73. A method of increasing the numbers of CD8.sup.+ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising the cell of claim 25, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific CD8.sup.+ CTLs.

74. A method of increasing the numbers of CD8.sup.+ CTLs specific for a selected desired antigen in a subject comprising administering an effective amount of a composition comprising the chimeric polypeptide of claim 29, wherein the antigenic peptide comprises an epitope that binds to and is presented on the cell surface by MHC class I proteins, thereby increasing the numbers of antigen-specific CD8.sup.+ CTLs.

78. A method of inhibiting the growth of a tumor in a subject comprising administering an effective amount of a composition comprising the cell of claim 25, thereby inhibiting growth of the tumor.

6. Document ID: US 20040071736 A1

L13: Entry 6 of 32 File: PGPB Apr 15, 2004

DOCUMENT-IDENTIFIER: US 20040071736 A1
TITLE: Methods and compounds for the treatment of mucus hypersecretion

CLAIMS:

1. A method of treating hypersecretion of mucus, comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising:--(a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin; and wherein, following administration to said patient, the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

2. A method according to claim 1, wherein said translocating domain is a translocating domain of a microbial protein.

3. A method according to claim 1, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

4. A method according to claim 1, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

5. A method according to claim 1, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus

haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

7. A method according to claim 1, wherein said targeting domain binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, serous cells, sensory efferent C-fibres, and Non-adrenergic Non-Cholinergic neural system fibres.

8. A method of treating chronic obstructive pulmonary disease (COPD), comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising:--(a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain of that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin; and wherein following administration to said patient the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

9. A method according to claim 8, wherein said translocating domain is a translocating domain of a microbial protein.

10. A method according to claim 8, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

11. A method according to claim 8, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

12. A method according to claim 8, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

14. A method according to claim 8, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

15. A method for treating asthma, comprising administering, topically to the airways of a patient in need thereof, a therapeutically effective amount of a compound, said compound comprising:--(a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that binds to a target cell selected from the group consisting of (i) a mucus secreting cell, and (ii) a neuronal cell controlling or directing mucus secretion; and (c) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin; and wherein following administration to said patient the compound binds to and delivers the L-chain or L-chain fragment into said target cell, thereby (i) inhibiting mucus

secretion by mucus secreting cells, (ii) inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion, or (iii) inhibiting mucus secretion by mucus secreting cells and inhibiting neurotransmitter release from neuronal cells controlling or directing mucus secretion.

16. A method according to claim 15, wherein said translocating domain is a translocating domain of a microbial protein.

17. A method according to claim 15, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

18. A method according to claim 15, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

19. A method according to claim 15, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

21. A method according to claim 15, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

22. A compound which inhibits mucus secretion by mucus secreting cells, said compound comprising:--(a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (b) a targeting domain that selectively binds to a target cell that is a mucus secreting cell; and (c) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin.

23. A compound according to claim 22, wherein said translocating domain is a translocating domain of a microbial protein.

24. A compound according to claim 22, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

25. A compound according to claim 22, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

26. A compound according to claim 22, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

28. The compound according to claim 22, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

29. A compound according to claim 22, wherein said targeting domain binds to (i) a mucus secreting cell, but not to (ii) a neuronal cell controlling or directing mucus secretion.

31. A compound according to claim 29, wherein said targeting domain binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

32. A pharmaceutical composition for topical administration to airways of a patient suffering from mucus hypersecretion, comprising:--(a) an amount of a compound, effective to inhibit mucus hypersecretion, wherein the compound comprises:--(i) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (ii) a targeting domain that selectively binds to a target cell that is a mucus secreting cell; and (iii) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; with the proviso that said compound is not a botulinum toxin; and (b) a formulation component selected from the group consisting of an excipient, an adjuvant and a propellant; wherein the composition is for nasal or oral administration of the compound to a patient.

33. A pharmaceutical composition according to claim 32, wherein said translocating domain is a translocating domain of a microbial protein.

34. A pharmaceutical composition according to claim 32, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

35. A pharmaceutical composition according to claim 32, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

36. A pharmaceutical composition according to claim 32, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

38. A pharmaceutical composition according to claim 32, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

40. A method of manufacture of a compound according to claim 22, comprising:--(a) obtaining a clostridial neurotoxin and removing or disabling the native target cell binding domain (H.sub.C) and native translocation domain (H.sub.N) of said clostridial neurotoxin to produce a modified clostridial neurotoxin or (b) obtaining a modified clostridial neurotoxin that has had the native target cell binding domain (H.sub.C) and the native translocation domain (H.sub.N) removed or disabled; and (c) linking the modified neurotoxin with:--(i) a targeting domain that selectively binds the compound to a mucus secreting cell, and (ii) a translocating domain that translocates the L-chain or L-chain fragment into the target cell.

41. A method according to claim 40, wherein said translocating domain is a translocating domain of a microbial protein.

42. A method according to claim 40, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

43. A method according to claim 40, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

44. A method according to claim 40, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

46. A method according to claim 40, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

47. A method of manufacture of a compound according to claim 22, comprising linking together: (a) a light chain (L-chain) or L-chain fragment of a clostridial neurotoxin, which L-chain or L-chain fragment includes the active proteolytic enzyme domain of the L-chain; (b) a translocating domain that translocates the L-chain or L-chain fragment into the target cell; and (c) a targeting domain that selectively binds the compound to a mucus secreting cell.

48. A method according to claim 47, wherein said translocating domain is a translocating domain of a microbial protein.

49. A method according to claim 47, wherein said translocating domain is a translocating domain of a bacterial or viral protein.

50. A method according to claim 47, wherein said translocating domain is a translocating domain of a bacterial toxin, or a translocating domain of a virally expressed membrane fusion protein.

51. A method according to claim 47, wherein said translocating domain is selected from the group consisting of a translocating domain of diphtheria toxin, domain II of pseudomonas exotoxin A, a translocating domain of influenza virus haemagglutinin, a translocating domain of a fusogenic protein of Semliki Forest virus, a translocating domain of vesicular stomatitis virus glycoprotein G, a translocating domain of SER virus F protein and a translocating domain of Foamy virus envelope glycoprotein.

53. A method according to claim 47, wherein said targeting domain selectively binds to a target cell selected from the group consisting of epithelial goblet cells, submucosal gland mucus-secreting cells, Clara cells, and serous cells.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Sequences | Attachments | Claims | KWIC | Draw De

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☐ 7. Document ID: US 20040071731 A1

L13: Entry 7 of 32 File: PGPB Apr 15, 2004

DOCUMENT-IDENTIFIER: US 20040071731 A1
TITLE: Chimeric protein comprising non-toxic pseudomonas exotoxin a and type iv pilin sequences

CLAIMS:

1. A chimeric protein comprising: a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin A sequence, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and further wherein the chimeric protein, when introduced into a host, is capable of generating polyclonal antisera that reduce adherence of the microorganism expressing the Type IV pilin loop sequence to the epithelial cells.

2. The chimeric protein of claim 1, wherein the chimeric protein, when introduced into the host, is also capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

3. The chimeric protein of claim 1, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

4. The chimeric protein of claim 3, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

5. The chimeric protein of claim 4, wherein the Type IV pilin loop sequence is located between the translocation domain and the endoplasmic reticulum retention domain.

11. The chimeric protein of claim 5, wherein the translocation domain comprises amino acids 280 to 364 of domain II of Pseudomonas exotoxin A.

12. The chimeric protein of claim 5, wherein the translocation domain is domain II of Pseudomonas exotoxin A.

13. The chimeric protein of claim 5, wherein the endoplasmic reticulum retention domain is domain III of Pseudomonas exotoxin A except that amino acid Glu at position of 553 is deleted.

15. The chimeric protein of claim 5, wherein the cell recognition domain is domain Ia of Pseudomonas exotoxin A.

16. The chimeric protein of claim 5, wherein the cell recognition domain binds to .alpha.2-macroglobulin receptor, epidermal growth factor receptor, transferrin receptor, interleukin-2 receptor, interleukin-6 receptor, interleukin-8 receptor, Fc receptor, poly-IgG receptor, asialoglycoprotein receptor, CD3, CD4, CD8, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNFalpha receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, VEGF receptor, or cytokine receptor.

17. A chimeric protein comprising: (a) a non-toxic Pseudomonas exotoxin A sequence comprising domain Ia, domain II, and domain III; and (b) a Type IV pilin loop sequence, wherein the Type IV pilin loop sequence is located between domain II and domain III of the non-toxic Pseudomonas exotoxin A sequence.

18. The chimeric protein of claim 17, wherein the non-toxic Pseudomonas exotoxin A sequence has the amino acid sequence of SEQ ID NO:2 with .DELTA.E553.

21. A polynucleotide encoding a chimeric protein, the chimeric protein comprising: a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin

A sequence, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and further wherein the chimeric protein, when introduced into a host, is capable of generating polyclonal antisera that prevent adherence of the microorganism expressing the Type IV pilin loop sequence to the epithelial cells.

22. The polynucleotide of claim 21, wherein the chimeric protein, when introduced into the host, is also capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

23. The polynucleotide of claim 21, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

24. The polynucleotide of claim 23, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

25. The polynucleotide of claim 24, wherein the Type IV pilin loop sequence is located between the translocation domain and the endoplasmic reticulum retention domain.

31. The polynucleotide of claim 25, wherein the translocation domain comprises amino acids 280 to 364 of domain II of Pseudomonas exotoxin A.

32. The polynucleotide of claim 25, wherein the translocation domain is domain II of Pseudomonas exotoxin A.

33. The polynucleotide of claim 25, wherein the endoplasmic reticulum retention domain is domain III of Pseudomonas exotoxin A except that amino acid Glu at position of 553 is deleted.

34. The polynucleotide of claim 25, wherein the cell recognition domain is domain Ia of Pseudomonas exotoxin A.

35. The polynucleotide of claim 25, wherein the cell recognition domain binds to .alpha.2-macroglobulin receptor, epidermal growth factor receptor, transferring receptor, Fc receptor, poly-IgG receptor, asialoglycoprotein receptor, CD3, CD4, CD8, chemokine receptor, CD25, CD11B, CD11C, CD80, CD86, TNFalpha receptor, TOLL receptor, M-CSF receptor, GM-CSF receptor, scavenger receptor, VEGF receptor, or cytokine receptor.

36. A polynucleotide encoding a chimeric protein, the chimeric protein comprising: (a) a non-toxic Pseudomonas exotoxin A sequence comprising domain Ia, domain II, and domain III; and (b) a Type IV pilin loop sequence, wherein the Type IV pilin loop sequence is located between domain II and domain III of the non-toxic Pseudomonas exotoxin A sequence.

37. The polynucleotide of claim 36, wherein the non-toxic Pseudomonas exotoxin A sequence has the amino acid sequence of SEQ ID NO:2 with .DELTA.E553.

41. A cell comprising the expression cassette of claim 40.

42. A composition comprising a chimeric protein, the chimeric protein comprising: a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin A sequence, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and

further wherein the chimeric protein, when introduced into a host, is capable of generating polyclonal antisera that prevent adherence of the microorganism expressing the Type IV pilin loop sequence to the epithelial cells.

43. The composition of claim 42, wherein the chimeric protein, when introduced into the host, is also capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

46. The composition of claim 42, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

47. The composition of claim 46, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

49. A method for eliciting an immune response in a host, the method comprising the step of administering to the host an immunologically effective amount of a composition comprising a chimeric protein comprising: a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin A sequence, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and further wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that prevent adherence of the microorganism expressing the Type IV pilin loop sequence to the epithelial cells.

50. The method of claim 49, wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

52. The method of claim 49, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

53. The method of claim 52, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

55. A method of eliciting an immune response in a host, the method comprising the step of administering to the host an immunologically effective amount of an expression cassette comprising a polynucleotide encoding a chimeric protein comprising: a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin A, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and further wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that reduce adherence of the microorganism expressing the Type IV pilin loop sequence to the epithelial cells.

56. The method of claim 55, wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

58. The method of claim 55, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the

chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

59. The method of claim 58, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

61. A method of generating antibodies specific for a Type IV pilin loop sequence, comprising introducing into a host a composition comprising a chimeric protein comprising a non-toxic Pseudomonas exotoxin A sequence and a Type IV pilin loop sequence, the Type IV pilin loop sequence being located within the non-toxic Pseudomonas exotoxin A, wherein the chimeric protein is capable of reducing adherence of a microorganism expressing the Type IV pilin loop sequence to epithelial cells, and further wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that reduce adherence of the microorganism expressing the Type IV pilin loop sequence to epithelial cells.

62. The method of claim 61, wherein the chimeric protein, when introduced into the host, is capable of generating polyclonal antisera that neutralize cytotoxicity of Pseudomonas exotoxin A.

64. The method of claim 61, wherein the non-toxic Pseudomonas exotoxin A sequence comprises: (a) a translocation domain sufficient to effect translocation of the chimeric protein to a cell cytosol; and (b) an endoplasmic reticulum retention domain that functions to translocate the chimeric protein from endosome to endoplasmic reticulum.

65. The method of claim 64, wherein the chimeric protein further comprises a cell recognition domain that functions as a ligand for a cell surface receptor and that mediates binding of the chimeric protein to a cell.

☐ 8. Document ID: US 20030124147 A1

L13: Entry 8 of 32 File: PGPB Jul 3, 2003

DOCUMENT-IDENTIFIER: US 20030124147 A1
TITLE: DTAT fusion toxin

CLAIMS:

1. A method for killing a tumor cell, comprising contacting said tumor cell with a fusion toxin comprising the toxin domain of diphtheria toxin and a urokinase-type plasminogen activator domain.

2. The method of claim 1, wherein said tumor cell is a brain tumor cell.

5. The method of claim 1, wherein said tumor cell expresses the urokinase-type plasminogen activator receptor.

6. The method of claim 1, wherein contacting said tumor cell occurs in vivo.

7. The method of claim 1, wherein said fusion toxin comprises the translocation enhancer region of diphtheria toxin.

9. The method of claim 1, wherein said urokinase-type plasminogen activator domain is capable of binding to urokinase-type plasminogen activator receptor.

11. The method of claim 1, wherein said fusion toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of urokinase-type plasminogen activator.

12. A method for killing a glioblastoma tumor cell, comprising contacting said glioblastoma tumor cell with a fusion toxin comprising a urokinase-type plasminogen activator domain.

13. The method of claim 12, wherein said fusion toxin comprises a toxin domain of a toxin selected from the group consisting of diphtheria toxin, ricin, Pseudomonas exotoxin, colicin, anthrax toxin, tetanus toxin, botulinum neurotoxin, saporin, abrin, bryodin, pokeweed anti-viral protein, viscumin, and gelonin.

15. The method of claim 12, wherein said fusion toxin comprises an internalization domain of a toxin selected from the group consisting of diphtheria toxin, colicin, delta-Endotoxin, anthrax toxin, tetanus toxin, botulinum toxin, and Pseudomonas exotoxin.

16. The method of claim 12, wherein said fusion toxin comprises the translocation enhancing region of diphtheria toxin.

17. The method of claim 12, wherein said urokinase-type plasminogen activator domain is capable of binding to urokinase-type plasminogen activator receptor.

19. The method of claim 12, wherein said glioblastoma tumor cell expresses the urokinase-type plasminogen activator receptor.

20. The method of claim 12, wherein said fusion toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of the urokinase-type plasminogen activator.

22. The fusion toxin of claim 21, wherein said fusion toxin further comprises the translocation enhancing region of diphtheria toxin.

24. The fusion toxin of claim 21, wherein said toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of urokinase-type plasminogen activator.

29. A host cell comprising the vector of claim 28 and expressing a fusion toxin, said fusion toxin comprising the toxin domain of,diphtheria toxin and a urokinase-type plasminogen activator domain.

☐ 9. Document ID: US 20030054012 A1

L13: Entry 9 of 32 File: PGPB Mar 20, 2003

DOCUMENT-IDENTIFIER: US 20030054012 A1

TITLE: PSEUDOMONAS EXOTOXIN A-LIKE CHIMERIC IMMUNOGENS FOR ELICITING A SECRETORY IGA-MEDIATED IMMUNE RESPONSE

CLAIMS:

1. A method of eliciting a secretory IgA-mediated immune response in a subject comprising the step of administering to at least one mucosal surface of the subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a foreign epitope; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

11. The composition of claim 8 produced by administering to at least one mucosal surface of a subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a an epitope of HIV-1; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

☐ 10. Document ID: US 20020165126 A1

L13: Entry 10 of 32 File: PGPB Nov 7, 2002

DOCUMENT-IDENTIFIER: US 20020165126 A1
TITLE: Method for inactivating gonadotrophs

CLAIMS:

1. A method for sterilizing a mammal, said method comprising administering an effective amount of a conjugate compound comprised of a peptide hormone selected from the group consisting of a GNRH analog (GnRH-A), a human chorionic gonadotropin, an equine chorionic gonadotropin, a luteinizing hormone or a follicle--stimulating hormone which is conjugated to a toxin selected from the group consisting of those plant toxins or bacterial toxins having a toxic domain and a translocation domain and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct chemical attack upon cells of said pituitary gland.

2. A method for sterilizing a mammal, said method comprising administering an effective amount of a conjugate compound comprised of a peptide hormone selected from the group consisting of a GnRH analog (GnRH-A), a human chorionic gonadotropin, an equine chorionic gonadotropin, a luteinizing hormone and a follicle--stimulating hormone which is conjugated to a toxin selected from the group consisting of those plant toxins: ricin, modeccin, abrin, pokeweed anti-viral

protein, a-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; those bacterial toxins: diphtheria toxin, pseudomonas exotoxin and shiga toxins having a toxic domain and a translocation domain or those chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin which are capable of direct chemical attack upon cells of said pituitary gland when conjugated to said peptide hormone.

3. A method for sterilizing a mammal, said method comprising administering an effective amount of a conjugate compound having the general formula: 4wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine; Y is a linking agent selected from the group consisting of: 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)ca- rbodimide (EDC), bis-diazobenzidine or glutaraldehyde; Z is a substituent selected from the group consisting of Gly-NH.sub.2, ethylamide, and AzA-Gly-NH.sub.2 and T is a toxin selected from the group consisting of those plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, a-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; those bacterial toxins: diphtheria toxin, pseudomonas exotoxin and shiga toxin having a toxic domain and a translocation domain or those chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct attack upon cells of said pituitary gland.

4. A method for sterilizing a mammal, said method comprising administering an effective amount of a conjugate compound having the formula: 5wherein Y is a linking agent selected from the group consisting of: 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)ca- rbodimide (EDC), bis-diazobenzidine or glutaraldehyde and T is a bacteria toxin selected from the group consisting of those diphtheria toxins, pseudomonas exotoxins and shiga toxins having a toxic domain and a translocation domain and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct attack upon cells of said pituitary gland.

5. A method for treating a sex hormone related disease in a mammal, said method comprising 30 administering an effective amount of a conjugate compound comprised of a peptide hormone selected from the group consisting of a GnRH analog (GnRH-A), a human chorionic gonadotropin, an equine chorionic gonadotropin, a luteinizing hormone or a follicle--stimulating hormone which is conjugated to a toxin selected from the group consisting of those plant toxins or bacterial toxins having a toxic domain and a translocation domain and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct chemical attack upon cells of said pituitary gland.

6. A method for sterilizing a mammal, said method comprising administering an effective amount of a conjugate compound comprised of a peptide hormone selected from the group consisting of a GnRH analog (GnRH-A), a human chorionic gonadotropin, an equine chorionic gonadotropin, a luteinizing hormone and a follicle--stimulating hormone which is conjugated to a toxin selected from the group consisting of those plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, a-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; those bacterial toxins: diphtheria toxin, pseudomonas exotoxin and shiga toxins having a toxic domain and a translocation

domain or those chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin which are capable of direct chemical attack upon cells of said pituitary gland when conjugated to said peptide hormone.

7. A method for treating a sex hormone related disease in a mammal, said method comprising administering an effective amount of a conjugate compound having the general formula: 6wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine; Y is a linking agent selected from the group consisting of: 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)ca- rbodimide (EDC), bis-diazobenzidine or glutaraldehyde; Z is a substituent selected from the group consisting of Gly-NH.sub.2, ethylamide, and AzA-Gly-N.sub.2 and T is a toxin selected from the group consisting of those plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, a-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; those bacterial toxins: diphtheria toxin, pseudomonas exotoxin and shiga toxin having a toxic domain and a translocation domain or those chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct attack upon cells of said pituitary gland.

8. A method for treating a sex hormone related disease in a mammal, said method comprising administering an effective amount of a conjugate compound having the formula: 7wherein Y is a linking agent selected from the group consisting of: 2-iminothiolane, N-succinimidyl-3-(2-pyridyldith- io) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)- -toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)ca- rbodimide (EDC), bis-diazobenzidine or glutaraldehyde and T is a bacteria toxin selected from the group consisting of those diphtheria toxins, pseudomonas exotoxins and shiga toxins having a toxic domain and a translocation domain and wherein said conjugate compound is capable of binding with a receptor cell of the mammal's pituitary gland and direct attack upon cells of said pituitary gland.

☐ 11. Document ID: US 20020142000 A1

L13: Entry 11 of 32 File: PGPB Oct 3, 2002

DOCUMENT-IDENTIFIER: US 20020142000 A1
TITLE: Anti-CD3 immunotoxins and therapeutic uses therefor

CLAIMS:

1. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof comprising a CD3-binding domain and a Pseudomonas exotoxin (PE) mutant, said PE mutant having ADP-ribosylating and translocation functions but substantially diminished cell-binding ability.

2. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 1 wherein the CD3-binding domain comprises an anti-CD3 antibody or CD3-binding fragment thereof.

3. A recombinant immunotoxin polypeptide polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the anti-CD3 antibody or CD3-binding fragment thereof binds an epitope on the .epsilon. chain of human CD3.

4. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the anti-CD3 antibody or CD3-binding fragment thereof binds an epitope formed by the .epsilon. and .gamma. chains of human CD3.

5. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the CD3-binding domain comprises a Fab fragment of an anti-CD3 antibody.

6. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the CD3-binding domain comprises the Fv region, or a CD3-binding fragment thereof, of an anti-CD3 antibody.

7. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the CD3-binding domain comprises monoclonal antibody UCHT-1 or a CD3-binding fragment thereof.

8. A recombinant immunotoxin polypeptide polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the CD3-binding domain comprises the Fv region, or a CD3-binding fragment thereof, of an antibody selected from: monoclonal antibody UCHT-1, an antibody having a variable region which is at least 80% identical to the variable region of UCHT-1, an antibody having complementarity-determining regions identical with those of UCHT-1 and having at least one sequence segment of at least five amino acids of human origin, and an antibody competing with UCHT-1 for binding to human CD3 antigen at least about 80% as effectively on a molar basis, and having at least one sequence segment of at least five amino acids of human origin.

9. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 2 wherein the CD3-binding domain comprises a single chain Fv of an anti-CD3 antibody.

11. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 10 wherein the CD3-binding domain comprises a single chain Fv of UCHT-1.

12. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 1 comprising a single chain Fv of UCHT-1 fused to a PE mutant essentially deleted of its cell-binding domain.

14. A recombinant immunotoxin polypeptide and pharmaceutically acceptable salts thereof according to claim 1 consisting essentially of the single chain Fv of an anti-human CD3 antibody fused via the carboxy terminus thereof to a PE mutant essentially deleted of its cell-binding domain.

21. A method for treatment or prophylaxis of T-cell mediated disorders in a patient comprising administering to a patient in need thereof a therapeutically effective amount of a recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt according to claim 1.

24. An autologous therapy for treating or preventing a T-cell mediated disorder or condition in a patient, comprising: (a) recruiting from the patient a cell population comprising CD3-bearing cells; (b) treating the cell population with a

recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt according to claim 1 to at least partially deplete said cell population of CD3-bearing cells; and (c) reinfusing the treated cell population into the patient.

25. A method for treatment or prophylaxis against graft versus host disease in patient to undergo a bone marrow transplant comprising: (a) providing an inoculum comprising isolated bone marrow and/or stem cell-enriched peripheral blood cells of a suitable donor treated with a T-cell depleting effective amount of a recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt according to claim 1; and (b) transplanting the inoculum into the patient.

26. A method for the treatment or prophylaxis or treatment of transplant rejection in a patient to undergo a bone marrow transplant comprising: (a) reducing the levels of viable CD3-bearing cell population in the patient; (b) providing an inoculum comprising isolated bone marrow and/or stem cell-enriched peripheral blood cells of a suitable donor treated with a T-cell depleting effective amount of a recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt according to claim 1; and (c) introducing the inoculum into the patient, and thereafter optionally administering a recombinant immunotoxin polypeptide according to claim 1 to the patient to further deplete donor and patient T cells.

27. A method of conditioning a patient to be transplanted with cells, or a tissue or organ of a donor, the method comprising: (a) depleting the CD3-bearing cell population in the patient; (b) providing an inoculum comprising isolated bone marrow and/or stem-cell enriched peripheral blood cells of the donor treated with a T-cell depleting effective amount of a recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt according to claim 1; (c) introducing the inoculum into the patient; and (d) transplanting the donor cells, tissue or organ into the patient.

28. A method according to claim 21 comprising co-administering the recombinant immunotoxin polypeptide or its pharmaceutically acceptable salt with at least one other pharmaceutical agent selected from cyclosporin A, rapamycin, 40-O-(2-hydroxy) ethyl rapamycin (RAD), FK-506, mycophenolic acid, mycophenolate mofetil (MMF), cyclophosphamide, azathioprene, leflunomide, mizoribine, a deoxyspergualine compound or derivative or analog, 2-amino-2-[2-(4-octylphenyl)ethyl]propane-1,3-diol, corticosteroids, anti-LFA-1 and anti-ICAM antibodies, and other antibodies that prevent co-stimulation of T cells.

☐ 12. Document ID: US 20020106370 A1

L13: Entry 12 of 32 File: PGPB Aug 8, 2002

DOCUMENT-IDENTIFIER: US 20020106370 A1
TITLE: IMPROVEMENTS IN OR RELATING TO PEPTIDE DELIVERY

CLAIMS:

1. A chimaeric polypeptide comprising: a binding portion comprising an immunoglobulin molecule or an effective portion thereof having specific binding affinity for a eukaryotic target cell surface component, and an effector portion

consisting of one or more copies of an immunogenic peptide; whereby binding of the polypeptide to the cell surface component induces internalisation of at least the effector portion so as to allow the immunogenic peptide to be presented by MHC molecules on the target cell surface:

2. A chemaric polypeptide comprising: a binding portion, from a first source, having specific binding affinity for a eukaryotic target cell surface component; an effector portion, from a second source, comprising a peptide capable of exerting a biological effect; and a translocation portion, from a third source, the translocation portion being adjacent to the effector portion; whereby binding of the polypeptide to the cell surface component induces internalisation of at least the effector and translocation portions so as to allow the effector portion to enter the cytosol of the target cell and thence allow the peptide to exert is biological effect.

3. A polypeptide according to claim 2, wherein the binding portion comprises an immunoglobulin molecule or an effective portion thereof.

5. A polypeptide according to any one of the preceding claims, wherein the cell surface component is an antigen or a receptor molecule.

6. A polypeptide according to one of the preceding claims, wherein after internalisation the peptide is presented on the surface of the target cell in association with class I MHC antigen so as to modulate a CTL response.

7. A polypeptide according to any one of claims 4, 5 or 6, as dependent on claim 1, wherein after internalisation the peptide is presented on the surface of the target cell in association with class II MHC antigen so as to modulate a T helper cell response.

8. A polypeptide according to any one of the preceding claims, wherein the effector portion comprises one or more immunodominant T cell peptide epitopes.

11. A polypeptide according to any one of the preceding claims, wherein the cell surface component is selected from the group consisting of: MHC class I antigen; MHC class II antigen; FcRI receptor; B cell surface immunoglobulin; Lewis Y antigen; TSH receptor; and the MBr1 antigen.

14. A polypeptide according to any one of the preceding claims, comprising a translocation portion derived from the translocation domain of a bacterial exotoxin or HIV tat protein, or the endosome-disrupting function of an adenovirus.

15. A polypeptide according to claim 14, wherein the signal is derived from the translocation domain of Pseudomonas exotoxin.

16. A polypeptide according to any one of the preceding claims, wherein the target cell is a "professional" antigen presenting cell (APC).

17. A polypeptide according to any one claims 1 to 15, wherein the target cell is an aberrant, virus-infected or otherwise diseased cell.

18. A polypeptide according to claim 17, wherein the target cell is a tumour cell.

19. A polypeptide according to claim 17 or 18, wherein the cell surface component is a tumour-associated antigen.

22. A method according to claim 21, wherein administering the polypeptide causes the target cell to present on its surface, together with an MHC antigen, an amino acid sequence which would not normally be presented by the target cell.

23. A method according to claim 22, wherein administering the polypeptide causes the target cell to present a CTL epitope which is foreign to the subject.

13. Document ID: US 6881718 B1

L13: Entry 13 of 32 File: USPT Apr 19, 2005

DOCUMENT-IDENTIFIER: US 6881718 B1
TITLE: Disulfide conjugated cell toxins and methods of making and using them

CLAIMS:

1. A method of making a cell toxin comprisng reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylatin domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

2. The method of claim 1, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located within ten amino acid residues of the translocation domain amino terminus.

3. The method of claim 1, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located at the translocation domain amino terminus.

4. The method of claim 1, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group is a cysteine residue.

5. The method of claim 1, wherein the Pseudomonas exotoxin translocation domain is covalently linked to the Pseudomonas exotoxin ADP-ribosylation domain.

6. The method of claim 5, wherein the covalent linkage between the Pseudomonas exotoxin translocation domain and the Pseudomonas exotin ADP-ribosylation domain is a peptide bond.

7. The method of claim 1, wherein the Pseudomonas exotoxin translocation domain . comprises an amino acid sequence as set forth in SEQ ID NO:1 and the Pseudomonas exotoxin ADP-ribosylation domain comprises an amino acid sequence as set forth in SEQ ID NO:2.

8. A pharmaceutical composition for the ablation of NK1 receptor expressing cells comprising a cell toxin and a pharmaceutically acceptable excipient, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide

comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

9. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located within ten amino acid residues of the translocation domain amino terminus.

10. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group located at the translocation domain amino terminus.

11. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain sulfhydryl group is a cysteine residue.

12. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain is covalently linked to the Pseudomonas exotoxin ADP-ribosylation domain.

13. The pharamceutical composition of claim 12, wherein the covalent linkage between the Pseudomonas exotoxin translocation domain and the Pseudomonas exotoxin ADP-ribosylation domain is a peptide bond.

14. The pharmaceutical composition of claim 8, wherein the Pseudomonas exotoxin translocation domain comprises an amino acid sequence as set forth in SEQ ID NO:1 and the Pseudomonas exotoxin ADP-ribosylation domain comprises an amino acid sequence as set forth in SEQ ID NO:2.

15. The pharmaceutical composition of claim 8, wherein the cell toxin and pharamceutically acceptable excipient are suitable for administration intrathecally, subdurally or directly into the brain parenchyma.

16. A method for ablating an NK1 receptor expressing cell in a patient comprising administering to said patient a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to ablate an NK1 receptor expressing cell, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

17. The method of claim 16, wherein the ablated NK1 receptor expressing cell is a dorsal horn cell, a stratum cell or a brain parenchyma cell.

18. A method of treating chronic pain without significantly afftecting basal nociceptive responses comprising administering to a subject in need thereof a cell toxin in a pharmaceutically acceptable excipient in an amount sufficient to treat chronic pain without significantly afftecting basal nociceptive responses, wherein the cell toxin is a substance P-Pseudomonas exotoxin disulfide linked conjugate

made by a process comprising the following steps: reacting a polypeptide comprising a Pseudomonas exotoxin translocation domain linked to a Pseudomonas exotoxin ADP-ribosylation domain, wherein the Pseudomonas exotoxin translocation domain comprises at least one reactive sulfhydryl group, with a substance P peptide comprising one additional cysteine residue at its amino terminal end, wherein the cysteine sulfhydryl group is disulfide linked to a di-thiobis (2-nitro)-benzoic acid group, such that after the reaction a disulfide bond is formed between the exotoxin polypeptide and the substance P and a thionitrobenzoate group is released, and purifying the substance P-Pseudomonas exotoxin disulfide linked conjugate from the released thionitrobenzoate group such that the purified conjugate is substantially free of thionitrobenzoate groups.

19. The method of claim 18, wherein cell toxin is administered to epineurium cells, perineurium cells, nerve ganglia, nerve sheathers, nerve linings, meninges, pia mater cells, arachnoid membrane cells, dura membrane cells, cells lining a joint or brain or spinal cord parenchymal cells.

☐ 14. Document ID: US 6846484 B2

L13: Entry 14 of 32 File: USPT Jan 25, 2005

DOCUMENT-IDENTIFIER: US 6846484 B2
TITLE: DTAT fusion toxin

CLAIMS:

1. A method for killing a tumor cell, comprising contacting said tumor cell with a fusion toxin comprising the toxin domain of diphtheria toxin and a urokinase-type plasminogen activator domain, wherein said contacting occurs in vivo, and wherein there exists an amount of said fusion toxin that: (a) does not result in life-threatening hepatic toxicity when said fusion toxin is administered subcutaneously; and (b) results in a decrease in the size of a tumor when said fusion toxin is administered into said tumor.

2. The method of claim 1, wherein said tumor cell is a brain tumor cell.

5. The method of claim 1, wherein said tumor cell expresses the urokinase-type plasminogen activator receptor.

6. The method of claim 1, wherein said fusion toxin comprises the translocation enhancer region of diphtheria toxin.

8. The method of claim 1, wherein said urokinase-type plasminogen activator domain is capable of binding to urokinase-type plasminogen activator receptor.

10. The method of claim 1, wherein said fusion toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of urokinase-type plasminogen activator.

11. A method for killing a glioblastoma tumor cell, comprising contacting said glioblastoma tumor cell with a fusion toxin comprising a urokinase-type plasminogen activator domain, wherein said contacting occurs in vivo, and wherein there exists an amount of said fusion toxin that: (a) does not result in life-threatening

hepatic toxicity when said fusion toxin is administered subcutaneously; and (b) results in a decrease in the size of a tumor when said fusion toxin is administered into said tumor.

12. The method of claim 11, wherein said fusion toxin comprises a toxin domain of a toxin selected from the group consisting of diphtheria toxin, ricin, Pseudomonas exotoxin, colicin, anthrax toxin, tetanus toxin, botulinum neurotoxin, saporin, abrin, bryodin, pokeweed anti-viral protein, viscumin, and gelonin.

14. The method of claim 11, wherein said fusion toxin comprises an internalization domain of a toxin selected from the group consisting of diphtheria toxin, colicin, delta-Endotoxin, anthrax toxin, tetanus toxin, botulinum toxin, and Pseudomonas exotoxin.

15. The method of claim 11, wherein said fusion toxin comprises the translocation enhancing region of diphtheria toxin.

16. The method of claim 11, wherein said urokinase-type plasminogen activator domain is capable of binding to urokinase-type plasminogen activator receptor.

18. The method of claim 11, wherein said glioblastoma tumor cell expresses the urokinase-type plasminogen activator receptor.

19. The method of claim 11, wherein said fusion toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of the urokinase-type plasminogen activator.

22. The pharmaceutical composition of claim 20, wherein said fusion toxin further comprises the translocation enhancing region of diphtheria toxin.

24. The pharmaceutical composition of claim 20, wherein said toxin comprises the toxin domain of diphtheria toxin, the translocation enhancing region of diphtheria toxin, and the amino-terminal fragment of urokinase-type plasminogen activator.

☐ 15. Document ID: US 6426075 B1

L13: Entry 15 of 32 File: USPT Jul 30, 2002

DOCUMENT-IDENTIFIER: US 6426075 B1
TITLE: Protease-activatable pseudomonas exotoxin A-like proproteins

CLAIMS:

1. A protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hour; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence

identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

2. The PE-like proprotein of claim 1 wherein the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:1) modified with amino acids substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280.

3. The PE-like proprotein of claim 1 wherein the protease activatable sequence is cleavable by a protease secreted by a cancer cell.

4. The PE-like proprotein of claim 1 wherein the cell recognition domain comprises an antibody that specifically binds to a cancer cell surface marker.

9. The PE-like proprotein of claim 3 wherein the cell recognition domain is coupled to the modified translocation domain through a peptide bond.

12. The PE-like proprotein of claim 8 wherein the cell recognition domain is an antibody coupled to the modified translocation domain through a peptide bond and wherein the antibody specifically binds a cancer cell surface marker.

13. A composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is substantially un-activatable by fibrin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ WD NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

14. The composition of claim 13, further comprising a PE Ib-like domain, wherein: (a) the cell recognition domain is an antibody coupled to the modified PE translocation domain through a peptide bond and wherein the antibody specifically binds a cancer cell surface marker; (b) the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:1) modified with amino acids substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280; and (c) the PE Ib-like domain, the cytotoxicity domain and the ER retention sequence together have the sequence of domains Ib and III of native PE.

16. A method for killing a cancer cell comprising contacting the cell with a protease-specific Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence cysteine-cysteine loop is substantially un-activatable by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic

reticulum ("ER") retention sequence.

17. The method of claim 16 wherein the cancer cell is a prostate cancer cell.

18. The method of claim 16 wherein the cancer cell is a colon cancer cell.

☐ 16. Document ID: US 6423513 B1

L13: Entry 16 of 32 File: USPT Jul 23, 2002

DOCUMENT-IDENTIFIER: US 6423513 B1
TITLE: Polynucleotides encoding protease-activatable pseudomonas exotoxin a-like proproteins

CLAIMS:

1. A recombinant polynucleotide comprising a nucleotide sequence encoding a protease-activatable Pseudomonas exotoxin A-like ("PE-like") proprotein comprising: (a) a cell recognition domain of between 10 and 1500 amino acids that binds to an exterior surface of a targeted cell; (b) a modified PE translocation domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 280 to 344 of SEQ ID NO:2 and which effects translocation to a cell cytosol upon proteolytic cleavage, wherein the translocation domain comprises a cysteine-cysteine loop that comprises a protease activatable sequence cleavable by a protease and wherein the protease activatable sequence is refractory to cleavage by furin when incubated with furin at a 1:10 enzyme:substrate molar ratio at 25.degree. C. for 16 hours; (c) a cytotoxicity domain comprising an amino acid sequence with 80% or greater sequence identity to amino acids 400 to 613 of SEQ ID NO:2, the cytotoxicity domain having ADP-ribosylating activity; and (d) an endoplasmic reticulum ("ER") retention sequence.

2. The recombinant polynucleotide of claim 1, further comprising a nucleic acid sequence encoding a PE Ib-like domain comprising an amino acid sequence of between 5 and about 1500 amino acids, which amino acid sequence is positioned between the modified PE translocation domain and the cytotoxicity domain and which does not interfere with the ability of the PE-like proprotein to bind cells, translocate, or ribosylate ADP.

4. The recombinant polynucleotide of claim 2 wherein: (a) the cell recognition domain is an antibody coupled to the modified PE translocation domain through a peptide bond and wherein the antibody specifically binds a cancer cell surface marker; (b) the modified PE translocation domain has a PE domain II sequence (amino acids 253-364 of SEQ ID NO:2) modified with amino acid substitutions introducing the protease activatable sequence so as to cause cleavage by the protease between amino acids 279 and 280: and (c) the PE Ib-like domain, the cytotoxicity domain and the ER retention sequence together have the sequence of domains Ib and III of native PE.

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw D

☐ 17. Document ID: US 6387684 B1

L13: Entry 17 of 32 File: USPT May 14, 2002

DOCUMENT-IDENTIFIER: US 6387684 B1
TITLE: Topoisomerase 1-mediated DNA delivery

CLAIMS:

1. A polypeptide consisting of, in any order, one or more ligand domains that specifically bind to a molecule on an external surface of a cell; one or more fragments of a Topoisomerase I, each fragment being less than 400 amino acids in length and consisting of an amino acid sequence corresponding to positions selected from the group consisting of 1-300, 51-200, 271-375, 422-596, and 651-696 of a Topoisomerase I; and one or more non-Topoisomerase I amino acid sequences.

2. The polypeptide of claim 1, wherein at least one of the ligand domains is a receptor binding domain of a Pseudomonas exotoxin A.

3. The polypeptide of claim 2, wherein at least one of the one or more non-Topoisomerase I amino acid sequences is a membrane translocation domain of a Pseudomonas exotoxin A.

8. The polypeptide of claim 3, wherein the receptor binding domain contains SEQ ID NO:5.

9. The polypeptide of claim 1, wherein at least one of the one or more non-Topoisomerase I amino acid sequences is a membrane translocation domain.

10. The polypeptide of claim 9, wherein the translocation domain is a membrane translocation domain of a Pseudomonas exotoxin A.

15. The polypeptide of claim 10, wherein the membrane translocation domain contains SEQ ID NO:6.

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw D

☐ 18. Document ID: US 6022950 A

L13: Entry 18 of 32 File: USPT Feb 8, 2000

DOCUMENT-IDENTIFIER: US 6022950 A
**** See image for Certificate of Correction ****
TITLE: Hybrid molecules having translocation region and cell-binding region

CLAIMS:

1. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding ligand effective to cause said hybrid molecule to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein each of said first part and said third part is non-native with respect to said naturally occurring protein, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond, provided that when said second part comprises a portion of a translocation domain of Pseudomonas exotoxin, said third part is not a polypeptide.

2. The hybrid molecule of claim 1, wherein said cell-binding ligand is a polypeptide.

9. The hybrid molecule of claim 2, wherein said first part comprises the binding domain of said cell-binding polypeptide ligand.

10. The hybrid molecule of claim 2, wherein said first part comprises the cell-binding polypeptide ligand.

11. The hybrid molecule of claim 2, wherein said cell-binding polypeptide ligand is a hormone.

12. The hybrid molecule of claim 2, wherein said cell-binding polypeptide ligand is an antigen-binding, single-chain analog of a monoclonal antibody.

14. The hybrid molecule of claim 2, wherein said first part comprises a portion of the binding domain of a polypeptide toxin.

16. The hybrid molecule of claim 4, wherein said polypeptide is an antigen-binding, single-chain analog of a monoclonal antibody.

26. The hybrid molecule of claim 20, wherein said toxin is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin, and Pseudomonas exotoxin A.

34. A hybrid molecule comprising a first part, a second part and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of the translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane and into the cytosol of the cell; and

(c) wherein said third part comprises a chemical entity to be introduced into the cell, wherein said chemical entity and said first part are non-native with respect to said diphtheria toxin, and further wherein said covalent bond connecting said second part and said third part is a cleavable bond.

35. The hybrid molecule of claim 34, wherein said first part comprises a portion of the binding domain of interleukin II effective to cause said hybrid molecule to bind to an interleukin II receptor-bearing cell.

36. The hybrid molecule of claim 34, wherein said first part comprises a portion of the binding domain of EGF.

49. A hybrid molecule comprising a first part, a second part, and a third part connected by covalent bonds,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of Shiga-like toxin which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises an enzymatically active domain of Shiga-like toxin.

50. The hybrid molecule of claim 49, wherein said cell-binding polypeptide ligand is interleukin-2.

☐ 19. Document ID: US 5965406 A

L13: Entry 19 of 32 File: USPT Oct 12, 1999

DOCUMENT-IDENTIFIER: US 5965406 A
**** See image for Certificate of Correction ****
TITLE: Recombinant DNAS encoding three-part hybrid proteins

CLAIMS:

1. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b).

2. The recombinant DNA molecule of claim 1, wherein said first part comprises the binding domain of said cell-binding polypeptide ligand.

3. The recombinant DNA molecule of claim 1, wherein said first part comprises the cell-binding polypeptide ligand.

4. The recombinant DNA molecule of claim 1, wherein said cell-binding polypeptide binding ligand is a hormone.

5. The recombinant DNA molecule of claim 1, wherein said cell-binding polypeptide ligand is an antigen-binding, single-chain analog of a monoclonal antibody.

7. The recombinant DNA molecule of claim 1, wherein said first part comprises a portion of the binding domain of a polypeptide toxin.

8. The recombinant DNA molecule of claim 1, wherein said polypeptide entity of (c) is an antigen-binding, single-chain analog of a monoclonal antibody.

14. The recombinant DNA molecule of claim 1, wherein said second part comprises a portion of the translocation domain of Shiga-like toxin.

18. A cell transformed with the recombinant DNA molecule of claim 1.

21. The recombinant DNA molecule of claim 12, wherein said toxin is selected from the group consisting of cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, diphtheria toxin, and Pseudomonas exotoxin A.

22. The recombinant DNA molecule of claim 15, wherein said first part comprises the binding domain of interleukin II.

23. The cell of claim 18, which is an E. coli cell.

29. A recombinant DNA molecule encoding a hybrid protein comprising a first part, a second part and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of the translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane and into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said polypeptide entity is non-native with respect to said diphtheria toxin.

30. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of interleukin II effective to cause said hybrid protein to bind to an interleukin II receptor-bearing cell.

31. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of diphtheria toxin.

32. The recombinant DNA molecule of claim 29, wherein said first part comprises a portion of the binding domain of EGF.

37. The recombinant DNA molecule of claim 29, wherein said cell binding polypeptide

ligand is non-native to said diphtheria toxin.

43. A cell transformed with the recombinant DNA molecule of claim 29.

44. The cell of claim 43, which is an E. coli cell.

45. A method of preparing a hybrid protein comprising a first part, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause said hybrid protein to bind to a cell of a animal;

(b) wherein said second part comprises a portion of a translocation domain of a naturally occurring protein selected from the group consisting of diphtheria toxin, botulinum neurotoxin, ricin, cholera toxin, LT toxin, C3 toxin, Shiga toxin, Shiga-like toxin, pertussis toxin and tetanus toxin, which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said naturally occurring protein of (b) comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule such that the hybrid protein is produced.

46. The method of claim 45, further comprising the step of harvesting the hybrid protein from the cells.

48. A method of preparing a hybrid protein comprising a first pail, a second part, and a third part,

(a) wherein said first part comprises a portion of the binding domain of a cell-binding polypeptide ligand effective to cause the hybrid protein to bind to a cell of an animal;

(b) wherein said second part comprises a portion of a translocation domain of diphtheria toxin which translocates said third part across the cytoplasmic membrane into the cytosol of the cell; and

(c) wherein said third part comprises a polypeptide entity to be introduced into the cell, wherein said third part is non-native with respect to said diphtheria toxin, comprising the steps of:

providing a cell transformed with a recombinant DNA molecule encoding the hybrid protein, and

culturing the transformed cell to allow expression of the recombinant DNA molecule such that the hybrid protein is produced.

49. The method of claim 48, further comprising the step of harvesting the hybrid protein from the cells.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

☐ 20. Document ID: US 5863745 A

L13: Entry 20 of 32 File: USPT Jan 26, 1999

DOCUMENT-IDENTIFIER: US 5863745 A
**** See image for Certificate of Correction ****
TITLE: Recombinant antibody-toxin fusion protein

CLAIMS:

1. A method for achieving targeted cytotoxicity, comprising contacting cells targeted to be killed with a cytotoxic amount of an antibody-PE40 recombinant fusion protein, wherein said antibody is a single-chain Fv fragment (scFv) and said PE40 is a Pseudomonas exotoxin (PE) fragment omitting amino acids 1 through 252 and possessing at least the translocating ard ADP ribosylating activity of PE, and wherein said cells targeted to be killed have receptors or antigens to which said antibody binds, a wherein said fusion protein has lower toxicity to ceus which lack receptors or antigens for the binding of said antibody.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw De

☐ 21. Document ID: US 5705163 A

L13: Entry 21 of 32 File: USPT Jan 6, 1998

DOCUMENT-IDENTIFIER: US 5705163 A
**** See image for Certificate of Correction ****
TITLE: Target-specific, cytotoxic, recombinant pseudomonas exotoxin

CLAIMS:

1. A method for killing a target cell, said method comprising contacting said target cell with a cytotoxic amount of a composition comprising a recombinant Pseudomonas exotoxin (PE) having a first recognition molecule for binding said target cell and a carboxyl terminal sequence of 4 to 16 amino acids which permits translocation of the PE molecule into a cytosol of said target cell, the first recognition molecule being inserted in domain III after and no acid 600 and before amino acid 613 of the PE.

2. A method of killing targeted cells, said method comprising the step of contacting cells targeted to be killed, with a cytotoxic amount of a recombinant Pseudomonas exotoxin fusion protein containing at least two different recognition molecules for killing cells expressing receptors to which said recognition molecules specifically bind.

10. The method of claim 4, wherein the first recognition molecule is an antibody or

a portion of an antibody which recognizes the target cell.

14. The method of claim 4, wherein a second recognition molecule is attached to the amino terminus of said Pseudomonas exotoxin.

18. The method of claim 2, wherein said two different recognition molecules comprise a first recognition molecule inserted in the carboxyl terminus of said Pseudomonas exotoxin, and a second recognition molecule attached to the amino terminus of said Pseudomonas exotoxin.

19. The method of claim 18, wherein said first recognition molecule is inserted in domain III after amino acid 600 and before amino acid 613 of said Pseudomonas exotoxin.

20. The method of claim 19, wherein said first recognition molecule is inserted in domain III after amino acid 607 of said of said Pseudomonas exotoxin molecule.

☐ 22. Document ID: US 5696237 A

L13: Entry 22 of 32 File: USPT Dec 9, 1997

DOCUMENT-IDENTIFIER: US 5696237 A
**** See image for Certificate of Correction ****
TITLE: Recombinant antibody-toxin fusion protein

CLAIMS:

1. An antibody-PE40 recombinant fusion protein wherein said antibody is a single-chain Fv fragment (scFv) and said PE40 is a Pseudomonas exotoxin (PE) fragment omitting amino acids 1 through 252 possessing at least the translocating and ADP ribosylating activity of PE.

4. A composition comprising an effective amount of the fusion protein of claim 1 to kill cells bearing a receptor or an antigen to which the antibody binds, and a pharmaceutically acceptable carrier.

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

☐ 23. Document ID: US 5677274 A

L13: Entry 23 of 32 File: USPT Oct 14, 1997

DOCUMENT-IDENTIFIER: US 5677274 A
**** See image for Certificate of Correction ****

TITLE: Anthrax toxin fusion proteins and related methods

CLAIMS:

1. A method for targeting compounds having a desired biological activity not present on native anthrax lethal factor (LF) to a specific cell population, comprising:

a) administering to the cell population a first compound comprising a first protein consisting essentially of:

i) the translocation domain and the anthrax lethal factor (LF) binding domain of the native anthrax protective antigen (PA) protein, and

ii) a ligand domain that specifically binds the first protein to a target on the surface of the cell population to bind the first compound to said surface; and

b) administering to the resultant cell population a second compound comprising a fusion protein or conjugate consisting essentially of:

i) the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein, chemically attached to

ii) a biological activity-inducing polypeptide to bind the second compound to the first compound on the surface of the cell population, internalize the second compound into the cell population, and effect the activity of the polypeptide therein.

2. A method according to claim 1, wherein the anthrax protective antigen (PA) binding domain of said second compound comprises at least the first 254 amino acid residues but less than all of the amino acid residues of the anthrax lethal factor (SEQ. ID NO: 2).

4. A method according to claim 1, wherein said second compound comprises the anthrax protective antigen (PA) binding domain of the native anthrax lethal factor (LF) protein chemically attached to a polypeptide through a peptide bond.

9. The method of claim 5, wherein the polypeptide of said second compound is Pseudomonas exotoxin A (PE).

☐ 24. Document ID: US 5668255 A

L13: Entry 24 of 32 File: USPT Sep 16, 1997

DOCUMENT-IDENTIFIER: US 5668255 A
** See image for Certificate of Correction **
TITLE: Hybrid molecules having translocation region and cell-binding region

CLAIMS:

1. A hybrid molecule comprising a first part and a second part connected via a covalent bond,

(a) said first part comprising a portion of the binding domain of a cell binding ligand effective to cause said hybrid molecule to bind to a cell of an animal; and

(b) said second part comprising a portion of the translocation domain of a protein, provided that said hybrid molecule does not include an enzymatically active portion of said protein, and wherein said first part and said second part are not segments of the same naturally occurring protein.

2. The hybrid molecule of claim 1, wherein said cell-binding ligand is a polypeptide.

4. The hybrid molecule of claim 2, wherein said first part comprises the binding domain of said polypeptide cell-binding ligand.

5. The hybrid molecule of claim 2, wherein said first part comprises the cell-binding polypeptide ligand.

6. The hybrid molecule of claim 2, wherein said cell-binding polypeptide binding ligand is a hormone or growth factor.

7. The hybrid molecule of claim 2, wherein said cell-binding polypeptide ligand is an antigen-binding, single-chain analog of a monoclonal antibody.

9. The hybrid molecule of claim 2, wherein said first part comprises a portion of the binding domain of a polypeptide toxin.

20. The hybrid molecule of claim 3, wherein said toxin is Pseudomonas exotoxin A.

Full | Title | Citation | Front | Review | Classification | Date | Reference | | | Claims | KWIC | Draw De

25. Document ID: US 5631229 A

L13: Entry 25 of 32 File: USPT May 20, 1997

DOCUMENT-IDENTIFIER: US 5631229 A
TITLE: Method for inactivating gonadotrophs

CLAIMS:

1. A method for functionally inactivating gonadotrophs in the pituitary gland of an animal, comprising administering to said animal an effective amount of a hormone/toxin conjugate comprising a peptide hormone conjugated to a toxin group, wherein said conjugate is capable of selectively binding with receptors on said gonadotrophs to render said gonadotrophs essentially incapable of secreting gonadotropins, wherein said animal is not weakened or killed by said method.

8. A method of claim 1, wherein said toxin group is a modified toxin comprising a toxic domain and a translocation domain but lacking a functional toxin cell binding domain.

9. A method of claim 1, wherein said toxin group is a toxin selected from the group consisting of a chemical toxin, a single chain toxin, and a modified toxin having

an intrinsic toxic group and intrinsic translocation domain but lacking a functional intrinsic toxin cell binding domain.

10. A method of claim 9, wherein said modified toxin is selected from the group consisting of modified ricin toxins, modified modeccin toxins, modified abrin toxins, modified diphtheria toxins, modified Pseudomonas exotoxins and modified shiga toxins.

13. A method of claim 1, wherein said toxin group is selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins, wherein said toxin group comprises a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

14. A method of claim 1, wherein said peptide hormone is conjugated to said toxin group using a linking agent capable of forming a stable conjugate that does not substantially degrade prior to binding with said gonadotrophs but which is capable of releasing a sufficient amount of said toxin group from said conjugate in the cytosol of said gonadotrophs to substantially preclude secretion of gonadotropins by said gonadotrophs.

19. A method of claim 1, wherein said conjugate has the general formula ##STR4## wherein Y comprises SMPB and wherein T is a toxin group selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins, wherein said toxin group comprises a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

21. The method of claim 1, wherein said conjugate can interact with GnRH receptors and gain entry into cells presenting said receptors.

22. A method for chemically attacking cells whose membranes contain receptors for GnRH, comprising administering to an animal an effective amount of a hormone/toxin conjugate comprising a peptide hormone that is capable of binding to a GnRH receptor, said peptide hormone conjugated to a toxin group, wherein said conjugate is capable of selectively binding to said GnRH receptors on said cells.

25. The method of claim 22, wherein said toxin group is a modified toxin comprising a toxic domain and a translocation domain but lacking a functional toxin cell binding domain.

26. The method of claim 22, wherein said toxin group is a toxin selected from the group consisting of a chemical toxin, a single chain toxin, and a modified toxin having an intrinsic toxic group and intrinsic translocation domain but lacking a functional intrinsic toxin cell binding domain.

27. The method of claim 22, wherein said conjugate has the general formula ##STR5## wherein Y comprises SMPB and wherein T is a toxin group selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins, wherein said toxin group comprises a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

☐ 26. Document ID: US 5587455 A

L13: Entry 26 of 32 File: USPT Dec 24, 1996

DOCUMENT-IDENTIFIER: US 5587455 A
TITLE: Cytotoxic agent against specific virus infection

CLAIMS:

1. A hybrid protein comprising a virus binding region from a cellular receptor sequence linked to a protein toxin sequence containing a region essential for cell toxicity, wherein said cellular receptor sequence is CD4.

2. The hybrid protein of claim 1 wherein the cellular receptor sequence is from CD4 and protein toxin sequence is from Pseudomonas exotoxin A.

3. A composition comprising the hybrid protein of claim 1 in an amount sufficient to kill a HIV-infected cell.

4. A hybrid protein according to claim 1 wherein the hybrid protein comprises a sequence of human CD4 containing a binding site for HIV linked to a cytotoxic protein, and wherein said hybrid protein binds to an HIV infected cell through said binding site, is internalized into and kills the HIV infected cell.

5. The hybrid protein of claim 2 wherein said protein is a recombinant fusion protein consisting of the first 178 amino acids of the CD4 cellular receptor and amino acids 1 to 3 and 253 to 613 of the Pseudomonas exotoxin A.

7. The hybrid protein according to claim 4 wherein the cytotoxic protein comprises a translocation and ADP-ribosylation domain.

8. The hybrid protein according to claim 4 wherein the cytotoxic protein is selected from the group consisting of Pseudomonas exotoxin A, diphtheria toxin fragment and ricin A.

Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw D

☐ 27. Document ID: US 5492893 A

L13: Entry 27 of 32 File: USPT Feb 20, 1996

DOCUMENT-IDENTIFIER: US 5492893 A
TITLE: Hormone-toxin conjugate compounds

CLAIMS:

1. A compound used for rendering gonadotrophs incapable of secreting gonadotropins, said compound having the general formula ##STR3## wherein T is a toxin group capable of precluding secretion of gonadotropin by said gonadotrophs; X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine; Y is a linking agent; and Z is a substituent selected from the group consisting of Gly-NH.sub.2, ethylamide, and AzA-Gly-NH.sub.2, said compound capable of being conveyed across a cell membrane of said gonadotrophs.

2. A compound of claim 1, wherein said toxin group is a modified toxin comprising a toxic domain and a translocation domain but lacks a functional toxin cell binding

domain.

3. A compound of claim 1, wherein Y is selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP),4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC),bis-diazobenzidine and glutaraldehyde; and T is a toxin group selected from the group consisting of ricin, modeccin, abrin, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof having at least a toxic domain, wherein said compound is capable of crossing the cell membrane of said gonadotrophs to substantially preclude secretion of hormones by said gonadotrophs.

4. A compound of claim 1, wherein X comprises D-lysine, wherein Y comprises SMPB, wherein Z comprises ethylamide, and wherein T is a toxin group selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins having a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

8. A hormone/toxin conjugate comprising a peptide hormone capable of binding to a GnRH receptor, conjugated to a toxin group, by a linking agent, said conjugate being capable of selectively binding to a gonadotroph and of substantially precluding said gonadotroph from secreting gonadotropins, said conjugate capable of being conveyed across a cell membrane of said gonadotrophs and said conjugate being capable, when administered in an effective amount to an animal, of sterilizing said animal without killing said animal.

10. A conjugate of claim 8, wherein said toxin group is selected from the group consisting of ricin, modeccin, abrin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof having at least a toxic domain.

11. A conjugate of claim 11, wherein said toxin group is a modified toxin comprising a toxic domain and a translocation domain but lacking a functional toxin cell binding domain.

14. A hormone/toxin conjugate comprising:

(a) a toxic domain of a toxin;

(b) a modified B-chain of a toxin that is capable of promoting translocation of said toxic domain across a cell membrane but is modified so as to be devoid of intrinsic binding activity;

(c) a hormone which is capable of selectively binding to a receptor on a gonadotroph;

(d) a linking agent that conjugates said toxin to said hormone; and

(e) whereby said conjugate, when administered to an animal in an effective amount, is capable of sterilizing said animal without killing said animal.

15. A hormone/toxin conjugate of claim 14, wherein said toxic domain is selected from the group consisting of ricin, modecoin, abrin, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, pokeweed antiviral protein, .alpha.-amanitin, gelonin

ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin toxic domains; and wherein said modified B-chain is selected from the group consisting of ricin, modeccin, abrin, diphtheria toxin, Pseudomonas exotoxin, and shiga toxin modified B-chains.

☐ 28. Document ID: US 5488036 A

L13: Entry 28 of 32 File: USPT Jan 30, 1996

DOCUMENT-IDENTIFIER: US 5488036 A
TITLE: Method for sterilizing animals using hormone-toxin conjugate compounds

CLAIMS:

1. A method for using at least one hormone/toxin conjugate to sterilize an animal, said conjugate comprising a peptide hormone capable of binding to a GnRH receptor, conjugated by a linking agent to a toxin group selected from the group consisting of a chemical toxin, a single chain toxin, and a modified toxin having an intrinsic toxic group lacking a functional binding domain, said conjugate capable of selectively binding to a gonadotroph and of substantially precluding said gonadotroph from secreting gonadotropins, said method comprising administering an effective amount of said conjugate to said animal to substantially preclude secretion of gonadotropins by said animal's gonadotrophs, wherein said conjugate is capable of crossing the cell membrane of a gonadotroph.

2. The method of claim 1, wherein said hormone/toxin conjugate has the formula ##STR3## wherein Y is a linking agent selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bis-diazobenzidine and glutaraldehyde; and T is a modified toxin selected from the group consisting of ricin toxins, modeccin toxins, abrin toxins, diphtheria toxins, Pseudomonas exotoxins and shiga toxins having a toxic domain and a translocation domain but lacking a functional toxin cell binding domain and wherein said conjugate is capable of selectively binding with receptors on gonadotrophs of said animal and substantially precluding secretion of gonadotropins by said gonadotrophs.

7. The method of claim 1, wherein said toxin group is a modified toxin comprising a toxic domain and a translocation domain but lacking a functional toxin cell binding domain.

8. The method of claim 1, wherein said modified toxin is selected from the group consisting of modified ricin toxins, modified modeccin toxins, modified abrin toxins, modified diphtheria toxins, modified Pseudomonas exotoxins and modified shiga toxins.

11. A method of claim 1, wherein said toxin group is selected from the group consisting of modified diphtheria toxins and modified Pseudomonas exotoxins,

wherein said toxin group comprises a toxic domain and a translocation domain but lacks a functional toxin cell binding domain.

15. A method for using at least one hormone/toxin conjugate to treat a sex hormone related disease in an animal, said sex hormone related disease selected from the group consisting of breast cancer, prostate cancer, sex-steroid dependent tumors, osteoporosis and endometriosis, said conjugate by a linking agent comprising a peptide hormone capable of binding to a GnRH receptor, conjugated to a toxin group capable of selectively binding to a gonadotroph and of substantially precluding said gonadotroph from secreting gonadotropins, said method comprising administering an effective amount of said conjugate to said animal to substantially preclude secretion of gonadotropins by said animal's gonadotrophs, wherein said conjugate is capable of crossing the cell membrane of a gonadotroph.

16. The method of claim 15, wherein said hormone/toxin conjugate has the formula ##STR4## wherein Y is a linking agent selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2pyridyldithio)propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide(EDC), bis-diazobenzidine and glutaraldehyde; and T is a modified toxin selected from the group consisting of ricin toxins, modeccin toxins, abrin toxins, diphtheria toxins, Pseudomonas exotoxins and shiga toxins having a toxic domain and a translocation domain but lacking a functional toxin cell binding domain and wherein said conjugate is capable of selectively binding with receptors on gonadotrophs of said animal and substantially precluding secretion of gonadotropins by said gonadotrophs.

18. A method for using at least one hormone/toxin conjugate to functionally inactivate cells whose membranes contain receptors for GnRH, said conjugate comprising a peptide hormone capable of binding to a GnRH receptor conjugated by a linking agent to a toxin group, said method comprising administering an effective amount of said conjugate to an animal to chemically attack said cells.

☐ 29. Document ID: US 5458878 A

L13: Entry 29 of 32 File: USPT Oct 17, 1995

DOCUMENT-IDENTIFIER: US 5458878 A
TITLE: P. exotoxin fusio proteins have COOHG220101al alterations which increase cytotoxicity

CLAIMS:

1. A fusion protein comprising a recombinant Pseudomonas exotoxin (PE) molecule, a first recognition moiety for binding a target cell, and a carboxyl terminal sequence of 4 to 16 residues which permits translocation of said fusion protein into the target cell cytosol, the first recognition moiety being inserted in domain III of PE after residue 600 and before residue 613.

9. The fusion protein of claim 2, wherein the first recognition molecule is an antibody or a portion of an antibody which recognizes the target cell.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a fusion protein comprising a recombinant Pseudomonas exotoxin (PE) molecule, a first recognition moiety for binding a target cell, and a carboxyl terminal sequence of 4 to 16 residues which permits translocation of said fusion protein into the target cell cytosol, the first recognition moiety being inserted in domain III of PE after residue 600 and before residue 613.

☐ 30. Document ID: US 5378688 A

L13: Entry 30 of 32 File: USPT Jan 3, 1995

DOCUMENT-IDENTIFIER: US 5378688 A
TITLE: GnRH analogs for destroying gonadotrophs

CLAIMS:

1. A method for sterilizing an animal, said method comprising administering an effective amount of a conjugate compound comprised of a gonadotropin releasing hormone (GnRH) or an analog thereof conjugated to a toxin selected from the group consisting of ricin, modecoin, abrin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof, wherein said conjugate compound is capable of crossing the cell membrane of a gonadotroph.

2. A method for sterilizing an animal, said method comprising administering an effective amount of a conjugate compound having the general formula ##STR6## wherein X is an amino acid selected from the group consisting of lysine, D-lysine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine, D-cysteine, tyrosine and D-tyrosine; Y is a linking agent selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bisdiazobenzidine and glutaraldehyde; Z is a substituent selected from the group consisting of Gly--NH.sub.2, ethylamide, and AzA--Gly--NH.sub.2 ; and T is a toxin selected from the group consisting of ricin, modeccin, abrin, pokeweed antiviral protein, .alpha.-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP, flax RIP, diphtheria toxin, Pseudomonas exotoxin, shiga toxin, melphalan, methotrexate, nitrogen mustard, doxorubicin, daunomycin, and modified forms thereof, wherein said conjugate compound is capable of selectively binding with receptors on gonadotrophs of said animal and crossing the cell membrane of said gonadotrophs.

3. A method for sterilizing an animal, said method comprising administering an effective amount of a conjugate compound having the formula ##STR7## wherein Y is a linking agent selected from the group consisting of 2-iminothiolane, N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), 4-succinimidyloxycarbonyl-.alpha.-(2-pyridyldithio)-toluene (SMPT), m-maleimidobenzoyl-N-hydroxysuccinimide

ester (MBS), N-succinimidyl(4-iodoacetyl) aminobenzoate (SIAB) , succinimidyl 4-(p-maleimidophenyl)butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), bis-diazobenzidine and glutaraldehyde; and T is a modified toxin selected from the group consisting of ricin toxins, modeccin toxins, abrin toxins, diphtheria toxins, Pseudomonas exotoxins and shiga toxins having a toxic domain and a translocation domain but lacking a functional toxin cell binding domain and wherein said conjugate compound is capable of selectively binding with receptors on gonadotrophs of said animal.

31. Document ID: US 5328984 A

L13: Entry 31 of 32 File: USPT Jul 12, 1994

DOCUMENT-IDENTIFIER: US 5328984 A
**** See image for Certificate of Correction ****
TITLE: Recombinant chimeric proteins deliverable across cellular membranes into cytosol of target cells

CLAIMS:

1. A chimeric protein of which a portion is translocated across a cellular membrane into the cytosol of target cells, the chimeric protein comprising, linked together at least (1) a first segment comprising a foreign protein desired to be introduced into the cytosol of the target cells, (2) a second segment from Domain II of Pseudomonas exotoxin having a translocation function which delivers the foreign protein across the cellular membrane into the cytosol of the target cells, and (3) a third segment which binds the chimeric protein to the target cells, the foreign protein being otherwise impermeable to the target cells and heterologous to the second segment.

2. The chimeric protein of claim 1, wherein said third segment is a ligand, an antibody, a growth factor or a cytokine for selective recognition of target cells.

6. A method for introducing a foreign protein across a cellular membrane into the cytosol of target cells, comprising the step of contacting cells into which a foreign protein is desired to be introduced, with the chimeric protein of claim 1.

8. A chimeric protein comprising:

a first segment comprising a foreign protein;

a second segment from Domain II of Pseudomonas exotoxin which translocates the first segment across a cellular membrane; and

a third segment which binds the chimeric protein to a target cell;

wherein the foreign protein is heterologous to the second segment.

10. The chimeric protein of claim 8, wherein the third segment is Domain Ia of Pseudomonas exotoxin.

12. A DNA molecule sequence that encodes a chimeric protein having a foreign protein segment, a segment from Domain II of Pseudomonas exotoxin that has a

translocation function which delivers the foreign protein across cellular membranes into the cytosol of target cells all linked to a third segment which encodes a protein that binds the chimeric protein to the target cells, the foreign protein being otherwise impermeable to the target cells and heterologous to the protein having the translocation function.

13. A method of making a translocatable chimeric protein, comprising the step of making a chimeric gene by linking together at least (1) a foreign protein gene sequence that encodes a foreign protein desired to be introduced into the cytosol of a target cell, (2) a heterologous gene sequence from a sequence encoding Domain II of Pseudomonas exotoxin that encodes a protein having a translocation function which delivers the foreign protein across the cellular membrane into the cytosol of the target cell, and (3) a gene sequence encoding a protein which binds the chimeric protein to the target cell, then allowing the expression of said chimeric gene in a suitable expression system so that a translocatable chimeric protein is obtained, and then recovering said chimeric protein from said expression system.

☐ 32. Document ID: US 4892827 A

L13: Entry 32 of 32 File: USPT Jan 9, 1990

DOCUMENT-IDENTIFIER: US 4892827 A
TITLE: Recombinant pseudomonas exotoxins: construction of an active immunotoxin with low side effects

CLAIMS:

1. A modified Pseudomonas exotoxin which comprises ADP ribosylating activity and the ability to translocate across a cell membrane; wherein the exotoxin comprises a deletion in the receptor binding domain Ia of the native toxin sufficient to render the modified toxin less toxic to human or animal cells in vitro and less toxic to the liver when administered in vivo relative to an unmodified Pseudomonas exotoxin.

2. The modified exotoxin of claim 1, being covalently bound to a cell recognition protein which binds to a receptor on the targeted cell membrane and selectively kills cells bearing said receptor.

3. A composition comprising a cytotoxic amount of the exotoxin of claim 2.

4. A method for achieving targeted cytotoxicity, comprising contacting cells targeted to be killed, with a cytotoxic amount of the composition of claim 3, said targeted cells being those having receptors to which said recognition protein binds, but the composition being without cytotoxicity to cells which comprise PE receptors and lack receptors for the cell recognition protein.

5. The modified exotoxin of claim 2 wherein said cell recognition protein is selected from the group consisting of an antibody, a peptide hormone, a growth factor and a cytokine.

6. The exotoxin of claim 5 wherein said cell recognition protein is an antibody.

7. The exotoxin of claim 5 wherein said growth factor is a-TGF.

8. The exotoxin of claim 5 wherein said cytokine is interleukin-2.

9. The exotoxin of claim 6 wherein said antibody is antitransferin receptor antibody.

10. The modified exotoxin of claim 1 produced by employing the plasmid of ATCC deposit number 67206, 67207, or 67208 under conditions permissive for expression of the toxin.

L4: Entry 1 of 7 File: PGPB Apr 14, 2005

DOCUMENT-IDENTIFIER: US 20050079171 A1
TITLE: Pseudomonas exotoxin A-like chimeric immunogens for eliciting a secretory IgA-mediated immune response

Summary of Invention Paragraph:
[0014] Third, because the Ib domain includes a cysteine-cysteine loop, epitopes that are so constrained in nature can be presented in near-native conformation. This assists in provoking an immune response against the native antigen. For example, a turn-turn-helix motif is evident with circular (constrained by a disulfide bond) but not linear peptides. (Ogata, M., et. al., 1990, Biol Chem 265, 20678-85.) Also, circular peptides are recognized more readily by anti-V3 loop monoclonal antibodies than linear ones. (Catasti, P., et. al., 1995, J Biol Chem 270, 2224-32.)

Detail Description Paragraph:
[0127] The non-native epitope domain can be linear or it can include a cysteine-cysteine loop that comprises the non-native epitope. In one embodiment, the non-native epitope domain includes a cysteine-cysteine loop that comprises the non-native epitope. This arrangement offers several advantages. First, when the non-native epitope naturally exists inside, or comprises, a cysteine-cysteine disulfide bonded loop, the non-native epitope domain will present the epitope in near-native conformation. Second, it is believed that charged amino acid residues in the native Ib domain result in a hydrophilic structure that sticks out away from the molecule and into the solvent, where it is available to interact with immune system components. Therefore, placing the non-native epitope within a cysteine-cysteine loop results in more effective presentation when the non-native epitope also is hydrophilic. Third, the Ib domain is highly insensitive to mutation. Therefore, although the cysteine-cysteine loop of the native Ib domain has only six amino acids between the cysteine residues, one can insert much longer sequences into the loop without disrupting cell binding, translocation, ER retention or ADP ribosylation activity.

L4: Entry 1 of 7 File: PGPB Apr 14, 2005

PGPUB-DOCUMENT-NUMBER: 20050079171
PGPUB-FILING-TYPE: new
DOCUMENT-IDENTIFIER: US 20050079171 A1

TITLE: Pseudomonas exotoxin A-like chimeric immunogens for eliciting a secretory IgA-mediated immune response

PUBLICATION-DATE: April 14, 2005

INVENTOR-INFORMATION:

NAME	CITY	STATE	COUNTRY	RULE-47
FitzGerald, David J.	Rockville	MD	US	
Mrsny, Randall J.	Redwood City	CA	US	

US-CL-CURRENT: 424/133.1

CLAIMS:

What is claimed is:

1. A method of eliciting a secretory IgA-mediated immune response in a subject comprising the step of administering to at least one mucosal surface of the subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a foreign epitope; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

2. The method of claim 1 wherein the mucosal surface is selected from mouth, nose, lung, gut, vagina, colon or rectum.

3. The method of claim 1 comprising administering a booster dose of the chimeric immunogen to a different mucosal surface.

4. The method of claim 1 further comprising administering to the subject a booster dose of the chimeric immunogen parenterally.

5. The method of claim 1 further comprising administering to the subject a booster dose of the chimeric immunogen to a mucosal surface.

6. The method of claim 1 further comprising administering to the subject a booster dose of the chimeric immunogen to a mucosal surface at least one year after an initial dose.

7. The method of claim 1 wherein the foreign epitope comprises a V3 loop apex of HIV-1.

8. A composition comprising secretory IgA antibodies that specifically recognize an epitope of HIV-1.

9. The composition of claim 8 wherein the foreign epitope comprises a V3 loop apex of HIV-1.

10. The composition of claim 8 wherein the foreign epitope is an epitope of herpes, vaccinia, cytomegalovirus, yersinia or vibrio.

11. The composition of claim 8 produced by administering to at least one mucosal surface of a subject a non-toxic Pseudomonas exotoxin A-like ("PE-like") chimeric immunogen comprising: (1) a cell recognition domain of between 10 and 1500 amino acids that binds to a cell surface receptor on the mucosal surface; (2) a translocation domain comprising an amino acid sequence substantially identical to a sequence of PE domain II sufficient to effect translocation to a cell cytosol; (3) a foreign epitope domain comprising an amino acid sequence of between 5 and 1500 amino acids that encodes a an epitope of HIV-1; and (4) an amino acid sequence encoding an endoplasmic reticulum ("ER") retention domain that comprises an ER retention sequence.

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L4: Entry 3 of 7 File: PGPB Oct 23, 2003

DOCUMENT-IDENTIFIER: US 20030198956 A1
TITLE: Staged assembly of nanostructures

Detail Description Paragraph:
[0160] For example, the known three-dimensional structure of many recombinant engineered components can serve as a guide for design of structural modifications to the antibody fragment that will enable the insertion of peptides (for example, at the site of a surface loop) that will confer novel binding, structural or functional properties to the antibody fragment. Moreover, there is a huge diversity of intermolecular specificities, such as that involving an antibody and a specific epitope, that can be either designed and constructed, or selected from a library. Advances in recombinant antibody technology have led to the creation of multivalent, multispecific and multifunctional antibodies (Chaudhary et al., 1989, A recombinant immunotoxin consisting of two antibody variable domains fused to Pseudomonas exotoxin, Nature 339(6223): 394-97; Neuberger et al. 1984, Recombinant antibodies possessing novel effector functions, Nature 312(5995): 604-08; Wallace et al., 2001, Exogenous antigen targeted to FcgammaRI on myeloid cells is presented in association with MHC class I, J. Immunol. Methods 248(1-2): 183-94) that may be used, according to the methods of the invention, as sources of structural elements and joining elements. Such multivalent, multispecific and multifunctional antibodies can be modified by the addition of functional groups for the construction of assembly units used for the fabrication of nanostructures as described herein.